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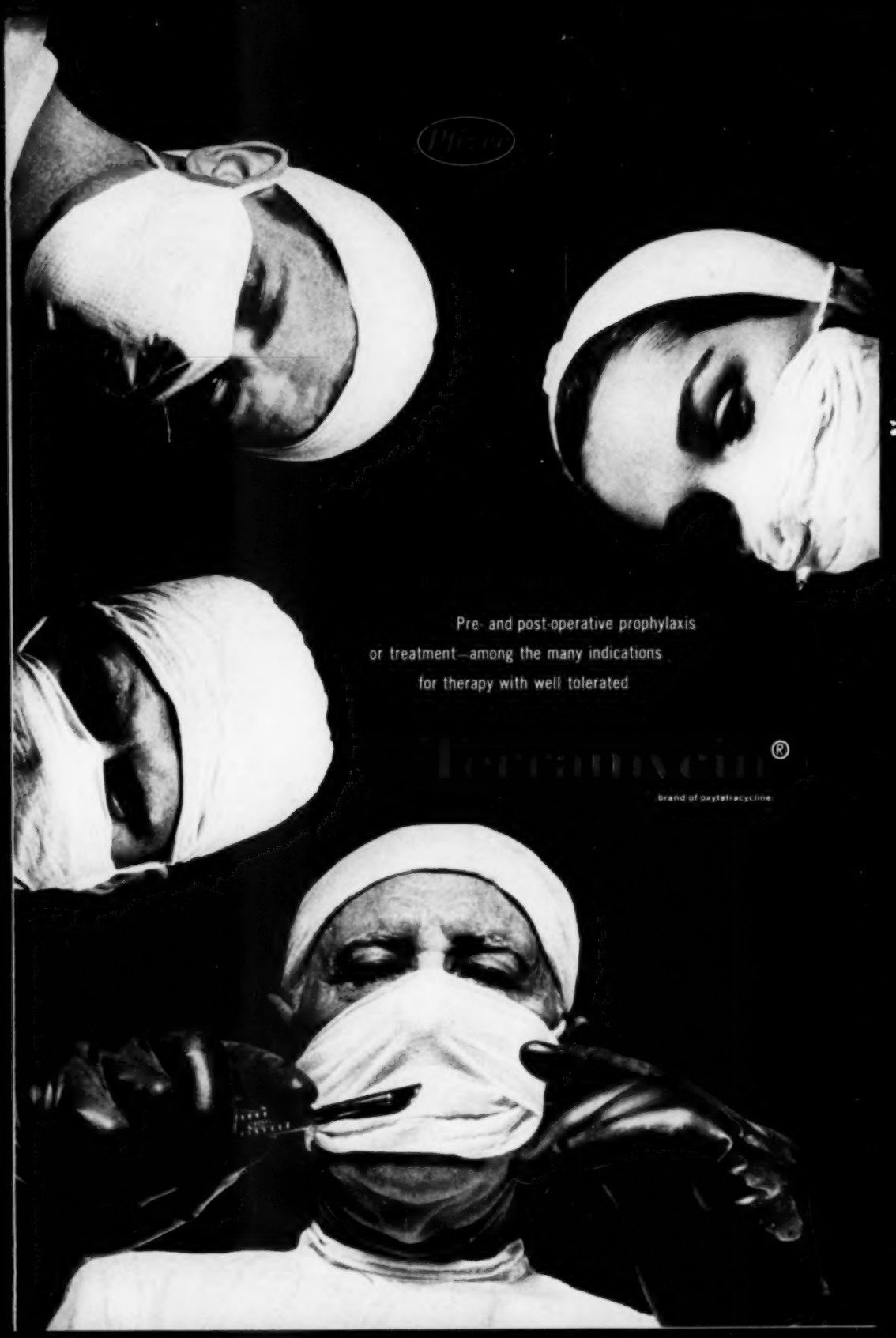
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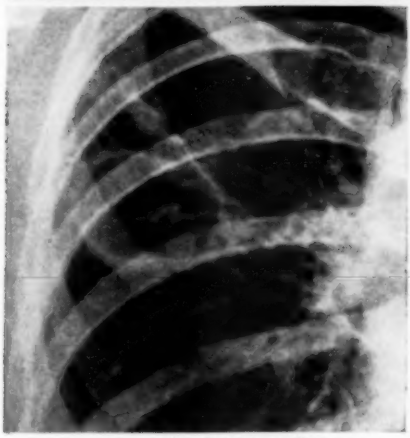

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THE AMERICAN REVIEW
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VOLUME 68 *September, 1953* NUMBER 3

OCCURRENCE OF HISTOPLASMOSIS IN EPIDEMICS[1,2]

Etiologic Studies

MICHAEL L. FURCOLOW AND J. THOMAS GRAYSTON

(Received for publication April 16, 1953)

INTRODUCTION

During the past decade, epidemics of pulmonary disease in which the etiology was obscure have been reported. These epidemics have acquired wide interest possibly because of some of the colorful names applied to them such as *cave sickness*, *acute miliary pneumonitis*, *granulomatous pneumonitis*, *angleworm pneumonia*, and *unusual pulmonary disease* (1–6). Examination for many potential etiologic agents had been unsuccessful. Perhaps the first to suggest that some of these epidemics might be caused by a fungus, possibly histoplasmosis, was Dr. Charles E. Smith, on the basis of some preliminary serologic tests which he performed in two of the epidemics (1, 5). Others, including the present writers, have been intrigued with the possibility that this series of epidemics and other similar unreported epidemics might be histoplasmosis. During a study of disseminated pulmonary calcification, White and Hill (7) found that 15 of the persons involved in the epidemic reported by Nauen and Korns (2) had developed widespread miliary calcifications and that all reacted to histoplasmin. Stored sera from this epidemic were examined then and found to contain complement-fixing antibodies for histoplasmosis. Recently, Loosli, Grayston, Alexander, and Tanzi (8) have reported the finding of an epidemic of pulmonary histoplasmosis in a farm family which was established by the isolation of the organism from one of the patients. The clinical and roentgenographic picture was similar to that of the aforementioned epidemics.

During the past three years, the present writers have carried out studies on a series of epidemics of disseminated pneumonitis, including the above, which have helped to establish the etiologic agent as *Histoplasma capsulatum*. It is the purpose here to report these etiologic studies. A companion paper (9) details the epidemiologic studies and presents data and conclusions regarding the natural reservoir of this fungus in nature and the method of its transmission to man.

[1] From the Communicable Disease Center, Public Health Service, Federal Security Agency, University of Kansas Medical Center, Kansas City, Kansas.

[2] Presented in part before the Medical Section, as part of the session on *Nontuberculous Chest Diseases*, at the annual meeting of the National Tuberculosis Association, Boston, Massachusetts, on May 26, 1952.

MATERIALS AND METHODS

A total of thirteen epidemics will be considered. In two of these epidemics (2, 3), efforts were confined to examination of soil samples from the point sources and surrounding areas. Detailed accounts of other studies in these two epidemics have appeared in one case (7) and in the other (10) may be expected shortly. Four of the other eleven epidemics being considered have been reported as pulmonary disease of unknown etiology (1, 5, 6, 11). Studies of these four epidemics were made possible by cooperation of the original authors or of the Armed Forces Epidemiological Board through Dr. John Dingle. In three of the seven unreported epidemics, the writers are indebted to the physicians who treated the patients for the privilege of studying the epidemics and for some of the data being reported (4, 12, 13).

In addition to ascertaining the similarity of the epidemics in their clinical and roentgenographic findings, four main types of etiologic studies were employed.

1. *Skin tests:* Histoplasmin, tuberculin, blastomycin, and coccidioidin skin tests were employed. One-tenth (0.1) ml. of the properly standardized antigen was injected intradermally. A positive reaction consisted in at least 5 mm. of induration at forty-eight hours.

2. *Serologic tests:* Most of the sera from these epidemics were examined by Dr. Joseph Schubert of the Communicable Disease Center, Public Health Service, using a complement fixation test for histoplasmosis with histoplasmin as antigen (14), and by Dr. Samuel Salvin of the Rocky Mountain Laboratory, Public Health Service, using both a complement fixation test with whole yeast phase antigen (15) and a precipitin test with histoplasmin as antigen (16). Some acute sera from early epidemics were examined by Dr. Daniel Tennenberg, then of the Kansas City Laboratory, Public Health Service, and by Dr. Charles Smith of the University of California. In the presentation detailing serologic findings, the grouping of results as positive, doubtful, or negative is based on the interpretation of the person performing the test. The most positive result obtained in the three tests is employed in the table summarizing serologic results.

3. *Follow-up chest roentgenograms:* In an effort to demonstrate calcification of the pulmonary lesions, roentgenograms of the chest were obtained as long after onset of illness and in as many of the persons involved in the epidemics as possible. The calcifications found were termed "disseminated miliary" when numerous small calcifications scattered uniformly throughout the lungs were seen and "single" or "multiple" when fewer, more localized, or irregular-shaped calcifications appeared.

4. *Cultures of soil for pathogenic fungi:* Soil collected from the place of infection of each epidemic was examined for the presence of pathogenic fungi. The method used in culture of the soil (17) consisted of inoculating soil suspended in saline intraperitoneally into laboratory white mice and culturing their livers and spleens three to four weeks later.

Clinical and Roentgenographic Characteristics of the Epidemic Illness

In order to emphasize the similarity of the epidemics one to another, a brief description of the clinical and roentgenographic findings is presented.

A strikingly consistent symptom complex accompanied the illnesses in all of the epidemics. Typically, the onset was sudden, with generalized malaise, followed in twelve to thirty-six hours by chills, fever, and prostration. Chest complaints were characteristically mild, with vague discomfort and nonproductive cough. Pronounced weakness was uniformly present, and occasionally dyspnea occurred.

Fever and acute illness lasted from a few days to two or three weeks. The weakness lasted much longer and was frequently a complaint for many months. A productive cough was often noted after the first week, but, in general, the physi-

FIG. 1

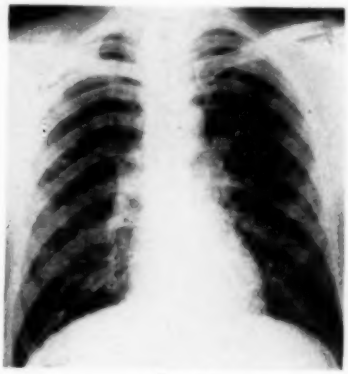

FIG. 2

FIG. 3

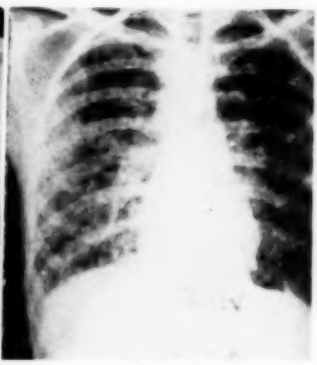

FIG. 4

FIG. 1. Plattsburg, New York, epidemic.
FIGS. 2 and 3. Camp Crowder, Missouri, epidemic.
FIG. 4. Camp Gruber, Oklahoma, epidemic.

cal findings and symptoms in the chest were remarkably few in relation to the extensive roentgenographic involvement.

The chest roentgenogram during the first week of illness might be normal, but the extensive bilateral involvement (figures 1 through 8) developed rapidly. The typical picture consisted of numerous and widely disseminated discrete infiltrations which varied in size from small, fine granular lesions (figure 5) through snowflake appearance (figure 7) to discrete nodules (figure 8). Involvement in

mild cases consisted of fewer lesions, which were sometimes localized. Hilar lymph node enlargement was usually present.

The roentgenographic abnormalities tended to clear in two to six months, although some lesions persisted, apparently in the form of generalized fibrosis.

FIG. 5

FIG. 6

FIG. 7

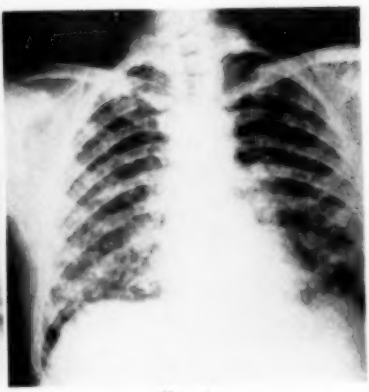

FIG. 8

FIG. 5. Cincinnati, Ohio, epidemic.
FIG. 6. Foreman, Arkansas, epidemic.
FIG. 7. Detroit Lakes, Minnesota, epidemic.
FIG. 8. Madison, Wisconsin, epidemic.

Whether apparent clearing occurred or not, a high percentage eventually (one to seven years) showed pulmonary calcification which was frequently of the disseminated miliary variety (figure 3).

Description of the Epidemics

A description of the place, date, persons involved, and activities associated with infection for each epidemic is presented in the order of occurrence.

In 1938, an epidemic occurred in *Plattsburg, New York*. This was described as *acute miliary pneumonitis* by Nauen and Korns (2). Twenty-three men who were engaged in shoveling pigeon manure from an old schoolhouse were involved. The illness in this epidemic tended to be severe. Of 10 men questioned ten years after the onset, 8 were still suffering from some pulmonary symptomatology. Figure 1 is an example of a chest roentgenogram of an acutely ill patient during this epidemic.

Camp Crowder, Missouri, had epidemics of pneumonitis in 1942 and 1943. The latter was reported as *atypical pneumonia* by Idstrom and Rosenberg (11), who stated that approximately 40 men from one training company were involved. Hospitalization records of 22 men were found, and follow-up studies were directed at this group. The illness in this epidemic tended to be mild. The chest roentgenograms showed moderate to marked generalized infiltration. Figures 2 and 3 are examples of acute and healed disseminated pneumonitis from this epidemic.

An epidemic occurred at *Camp Gruber, Oklahoma*, in 1944, and was reported as *an unusual pulmonary disease* by Cain, Devins, and Downing (5). This epidemic involved 35 soldiers in an army camp who sought shelter in a storm cellar during maneuvers. Epidemiologic studies showed that almost all of the men who entered the cellar became infected, and that no man became infected who had not entered the cellar. This epidemic caused a very severe illness, with 21 of the 35 men receiving disability discharges after seven months of hospitalization. Thirteen of 23 men, on whom information is available, are still receiving compensation. Roentgenograms of the lungs of all of the men involved in this epidemic showed the discrete disseminated infiltrations characteristic of these epidemics. Figure 4 shows an example of an acute-phase chest roentgenogram from this epidemic.

Disseminated pulmonary calcification in a twelve-year-old boy in *Topeka, Kansas*, was found to have originated during an acute respiratory illness six years previously, in 1944. At that time the boy was living on a farm. Study of his family and that of two other families who had occupied the farm subsequently revealed 4 cases of disseminated pulmonary calcification among the 11 members examined. These 4 cases consisted of a young boy in each family and another boy who had visited the farm and subsequently became ill. Four adults in these families showed evidence of single lesions or several calcified lesions in the lungs.

Nine days after moving into a farm house at *Williamsburg, Kansas*, in 1946, 3 of 9 members of the *B* family became ill. The illness was of a mild, febrile type in these 3 children but of a more severe nature in a fourth child who became ill a week later. Pulmonary infiltrations were present in the chest films of the 4 children and also in a fourteen-month-old infant who had not been noticeably ill. The oldest of the children who were ill was ten years of age. Two older children and the parents remained well. The roentgenographic shadows were of the generalized miliary type in the child who had the most severe illness. In the others single or several localized lesions with adenopathy were present.

An epidemic occurred in *Cincinnati, Ohio*, in 1947, and was reported by Feldman and Sabin as *pneumonitis of unknown etiology* (3). Twelve persons were infected at the time of shoveling a collection of pigeon manure from the basement of a water tower. The disease was mild to moderately severe, with the men recovering after several months of illness. The roentgenograms of all of the men showed disseminated pulmonary infiltration. Figure 5 is an example of a roentgenogram from this epidemic taken at the time of acute illness.

A family consisting of 2 grandparents, 6 parents, and 14 children participated in visiting and preparing for occupancy an old farm house on the edge of *Kansas City*,

Missouri, in July, 1947. The grandmother and 4 of the children became ill, one with a moderately acute illness. In addition, histoplasmin skin test conversion was demonstrated in the grandfather. Disseminated pulmonary lesions appeared in the most severely ill boy, and multiple lesions appeared in 2 other children. The other 3 persons showed lung infiltrations of lesser extent. The only time this particular group of 6 persons worked together was on July 7, at which time an old chicken coop was torn down.

An epidemic reported as *cave sickness* by Washburn, Tuohy, and Davis (1) occurred near *Foreman, Arkansas*, in 1947. This epidemic involved 25 men and boys who were excavating a cave on a treasure hunt. Illness in this epidemic was mild to moderately severe, with none of the men suffering long hospitalization. Chest roentgenograms again revealed the presence of typical disseminated pulmonary infiltrates. Figure 6 is an example of a chest film obtained during the acute illness of one person in this epidemic.

An unusual type of pulmonary disease involving 6 members of a family in *Detroit Lakes, Minnesota*, has been reported by Rutledge (6). All of the members in this farm family became ill during the same week in January, 1948. Epidemiologic evidence suggested that the only place all members of the family could have been infected together was in their farm house. The illness in 3 of these persons was mild, while in the other 3 it was quite severe. Four were examined roentgenographically at the time of acute illness and showed the typical disseminated infiltrates. At the time of follow-up studies, four years later, 2 of the 6 show definite beginning miliary calcification. An acute-phase chest roentgenogram of one of the severely ill patients is shown in figure 7.

An epidemic called *angleworm pneumonia* occurred in *Madison, Wisconsin*, in 1948. It is to be reported by Dickie and Ackerman (4). Ten persons were involved. The point source was a moist area on the edge of town where fishing worms were being dug. Chest roentgenograms in all of the men revealed typical disseminated pulmonary infiltrates. The illness in this epidemic tended to be mild to moderately severe. Follow-up roentgenograms obtained three years after onset of illness showed that calcification was developing in 3 of the patients. Figure 8 is an example of an acute-phase chest roentgenogram from this epidemic.

At *Bath, Illinois*, in April, 1951, 2 nine-year-old cousins became ill within a week of each other, three weeks after playing together in a hollow tree. The hollow-tree episode was well remembered because both boys became trapped in the tree due to its narrow opening, and the father of one of the boys had to bring a ladder to release them. The illness of both youngsters was moderately severe, one more so than the other. Both showed disseminated pulmonary infiltrations on roentgenographic examination.

A fatal case of histoplasmosis in a five-month-old infant occurred at *Bellevue, Iowa*, in September, 1951. Infection appeared to have been contracted at the home of the maternal grandparents during the first six weeks of the child's life. She was then taken to California, outside the known area of histoplasmin sensitivity, and returned to Iowa only three days before her illness developed. The grandparents and their two sons were the other inhabitants of the farm. Both of these sons had had a mild "flu-like" illness about the time the infant had been on the farm. Both of these boys showed disseminated fine infiltrations in the roentgenograms of the chest, while the grandfather showed healed multiple pulmonary calcification. The child's illness was of a progressive nature with enlargement of the liver and spleen, fever, anemia, and loss of weight.

In February, 1952, a severe respiratory illness occurred among 4 men about two weeks after they dismantled an old abandoned schoolhouse in *Mandan, North Dakota*. This was an extremely dusty job due to the large accumulation of pigeon and, possibly, bat excreta on the rafters and window casings. The illness was severe in all of the 4 men, the 2 who had more intimate exposure to the excreta having a more prolonged sickness. Chest roentgenograms of all 4 of the men showed disseminated infiltrations.

RESULTS

Skin tests: The results of skin testing 94 of the 116 persons involved in the eleven epidemics are summarized in table 1. All of those tested reacted positively

TABLE 1

RESULTS OF SKIN TESTS WITH HISTOPLASMIN, TUBERCULIN, BLASTOMYCIN, AND COCCIDIOIDIN IN PERSONS INVOLVED IN ELEVEN EPIDEMICS OF PULMONARY DISEASE

PLACE	YEAR	NUMBER OF PERSONS INVOLVED	TIME AFTER ILLNESS TESTED	SKIN TEST REACTIONS			
				Histoplasmin	Tuberculin	Blastomycin	Coccidioidin
Camp Crowder, Missouri	1943	22	9 years	10/10	3/10	3/10	1/8
Camp Gruber, Oklahoma	1944	26	7 years	25/25	9/25	4/25	2/25
Topeka, Kansas	1944–1950	4	2 to 6 years	4/4	0/4	0/1	0/1
Williamsburg, Kansas	1946	5	1 month	5/5	0/5	0/1	0/1
Kansas City, Missouri	1947	6	1 month	6/6	2/6	1/6	0/6
Foreman, Arkansas	1947	25	4 years	17/17	3/17	1/14	0/14
Detroit Lakes, Minnesota	1948	6	4 years	6/6	1/6	0/6	0/6
Madison, Wisconsin	1948	12	1 year	12/12	2/11	—	0/4
Bath, Illinois	1951	2	4 months	2/2	0/2	—	0/2
Bellevue, Iowa	1951	3	6 months	2/2	0/2	—	—
Mandan, North Dakota	1952	5	4 months	5/5	0/5	1/5	0/5
Totals		116		94/94	20/93	10/68	3/72

to histoplasmin. Only a small percentage of persons reacted to tuberculin, coccidioidin, or blastomycin.

To confirm the significance of finding 100 per cent positive histoplasmin skin tests, it is necessary to know what percentage of a similar "normal" population would be expected to react to histoplasmin. In figure 9 is shown the geographic location of the epidemics in relation to prevalence of histoplasmin sensitivity. Except for the two army camp epidemics, the persons involved in the epidemics were residents of the locale in which the outbreaks occurred. In the case of the soldiers infected at Camps Crowder and Gruber, investigation revealed that in practically all cases their place of major residence was in states of low histoplasmin sensitivity.

Age as well as residence will affect expected histoplasmin sensitivity, since children may have a much lower frequency of reactions than the adult prevalence which is shown in figure 9. Children were predominantly involved in three epidemics which occurred in areas of relatively high histoplasmin sensitivity. These

various facts tend to indicate that relatively few of the persons involved in the epidemics would have been expected to have positive skin test reactions to histoplasmin.

The demonstration of conversion of the histoplasmin skin test from negative to positive is evidence of recent *Histoplasma* infection. Fortunately, information on the status of the skin sensitivity to histoplasmin prior to illness was available on 3 children involved in the Kansas City epidemic. Routine skin testing of school children was being carried out in certain Kansas City schools, and these 3 children failed to react to histoplasmin in May prior to their illness in July. Positive reaction was demonstrated one to two weeks after the onset of illness. In addition, histoplasmin conversion was demonstrated in the grandfather of these children

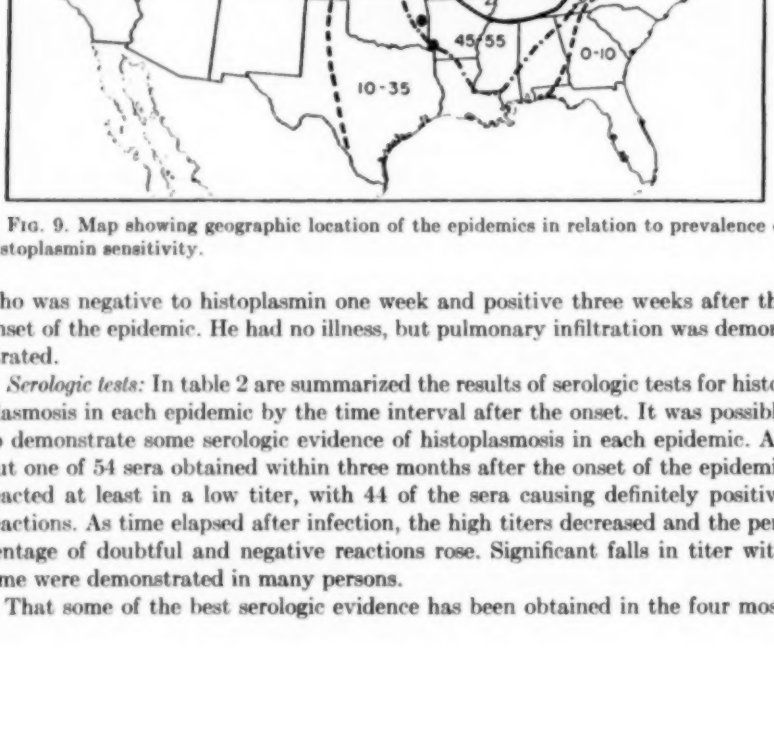

FIG. 9. Map showing geographic location of the epidemics in relation to prevalence of histoplasmin sensitivity.

who was negative to histoplasmin one week and positive three weeks after the onset of the epidemic. He had no illness, but pulmonary infiltration was demonstrated.

Serologic tests: In table 2 are summarized the results of serologic tests for histoplasmosis in each epidemic by the time interval after the onset. It was possible to demonstrate some serologic evidence of histoplasmosis in each epidemic. All but one of 54 sera obtained within three months after the onset of the epidemic reacted at least in a low titer, with 44 of the sera causing definitely positive reactions. As time elapsed after infection, the high titers decreased and the percentage of doubtful and negative reactions rose. Significant falls in titer with time were demonstrated in many persons.

That some of the best serologic evidence has been obtained in the four most

recently occurring epidemics is shown in table 2. This is due to availability of sera and improvement in serologic techniques in recent years.

The original publications of the serologic techniques used on these sera (14–16) emphasized the infrequent occurrence of what could be called "false positive" reactions. Observations of the present writers (18) who use these tests as a routine screening procedure have further confirmed their specificity.

Pulmonary calcification: The tendency of pulmonary histoplasmosis to heal with calcification has been demonstrated through numerous surveys (19, 20, 21). This type of healing has been found to be much more frequent with histoplasmosis than with other pulmonary mycotic infections or with tuberculosis. The

TABLE 2

RESULTS OF SEROLOGIC TESTS FOR HISTOPLASMOSIS* IN PERSONS INVOLVED IN ELEVEN EPIDEMICS OF PULMONARY DISEASE BY TIME INTERVAL AFTER ONSET OF ILLNESS

PLACE	YEAR	NUMBER OF PERSONS INVOLVED	TIME INTERVAL AFTER ONSET OF ILLNESS											
			1 to 3 Months			6 to 19 Months			2 to 4 Years			7 to 9 Years		
			Pos.	?	Neg.	Pos.	?	Neg.	Pos.	?	Neg.	Pos.	?	Neg.
Camp Crowder, Missouri	1943	22										4	0	6
Camp Gruber, Oklahoma	1944	26	25	2	0							3	11	6
Topeka, Kansas	1944–1950	4				0	0	2				0	1	0
Williamsburg, Kansas	1946	5				0	0	3	0	1	0			
Kansas City, Missouri	1947	6	4	2	0	1	1	1	0	2	3	0	1	0
Foreman, Arkansas	1947	25	3	5	1	1	9	2				0	3	15
Detroit Lakes, Minnesota	1948	6							1	3	2			
Madison, Wisconsin	1948	12	5	0	0	6	3	0	2	3	3			
Bath, Illinois	1951	2	1	0	0	1	1	0						
Bellevue, Iowa	1951	3	2	0	0	1	0	1						
Mandan, North Dakota	1952	5	4	0	0	4	1	0						
Totals		116	44	9	1	14	15	9	3	9	8	7	16	27

* *See* "Materials and Methods" section for details of tests and interpretations.

disseminated miliary type of calcification has been particularly associated with histoplasmosis. Almost all persons found with this unique type of pulmonary calcification have been histoplasmin reactors (7, 22, 23). The results of follow-up roentgenographic examinations of the chest of those involved in the epidemics are shown in table 3. Pulmonary calcification has been demonstrated in some of the persons from all of the epidemics which occurred prior to 1951. It may be seen in table 3 that, the longer the time interval since the occurrence of the epidemic, the larger the percentage of persons demonstrating calcified lung lesions. Of the 63 persons who exhibited some type of calcification, 44 had disseminated miliary calcification while the remaining 19 had single or multiple lesions. Only 12 of the 73 persons examined, who have been followed at least four years, failed to show calcification.

In regard to the inability to demonstrate much pulmonary calcification in the four most recent epidemics, it is worth while to reiterate that, in these four epidemics, especially complete serologic evidence was obtained.

Soil isolations: Examinations of soil samples collected at and around the point sources of the epidemics have yielded *H. capsulatum* from eleven of thirteen outbreaks. The isolations were from samples collected at the exact point source as listed above, under "*Description of the Epidemics*," except as follows:

Plattsburg, New York. The site of the schoolhouse, which was being torn down at the time of infection, has since been cemented over and a library building erected. Therefore, it was not possible to obtain a soil sample from the exact point source of the epidemic, but 24 soil samples from nearby locations and from other areas in Plattsburg were studied. One positive for *Histoplasma* was obtained from the dirt basement of a church two doors from the original site.

Camp Crowder, Missouri. Of 24 samples examined from the area of activity of the company involved, 3 yielded *Histoplasma*. These 3 samples came from in and around different buildings located along a 100-yard stretch of road.

Detroit Lakes, Minnesota. Of numerous samples from the vicinity of the farm which were cultured, one positive sample was found. This sample came from loose dirt underneath the kitchen floor where the warming stove was located.

Bellevue, Iowa. The one sample from this farm positive for *Histoplasma* came from along the edge of the farmhouse below the living-room window where the wood stove was located.

TABLE 3

THE FINDINGS OF PULMONARY CALCIFICATION IN FOLLOW-UP CHEST ROENTGENOGRAMS ON PERSONS INVOLVED IN ELEVEN EPIDEMICS OF PULMONARY DISEASE

PLACE	YEAR	NUMBER OF PERSONS INVOLVED	TIME INTERVAL TO LAST ROENTGENOGRAM	PULMONARY CALCIFICATION		
				Disseminated Miliary	Single or Multiple	None
Camp Crowder, Missouri	1943	22	9 years	7	3	0
Camp Gruber, Oklahoma	1944	26	7 years	20	2	3
Topeka, Kansas	1944–1950	4	2 to 7 years	4	0	0
Williamsburg, Kansas	1946	5	4 years	1	4	0
Kansas City, Missouri	1947	6	3 years	2	1	3
Foreman, Arkansas	1947	25	4 years	6	7	4
Detroit Lakes, Minnesota	1948	6	4 years	2	2	2
Madison, Wisconsin	1948	12	3 years	2	0	8
Bath, Illinois	1951	2	1 year	—	—	2
Bellevue, Iowa	1951	3	1 year	—	—	3
Mandan, North Dakota	1952	5	6 months	—	—	5
Totals		116		44	19	30

The two point sources from which *Histoplasma* has not as yet been isolated are the *Mandan, North Dakota*, schoolhouse site and the *Williamsburg, Kansas*, farm. In the latter case, a fungus grossly resembling *Histoplasma* was passed

through mice. However, its failure to develop the characteristic tuberculated spores has so far prevented identification.

It is emphasized that, with the exceptions noted above, the fungus was isolated from the exact point source of the epidemics (cave, storm-cellar, tower, et cetera) and not from samples taken from surrounding areas.

Other epidemics: In addition to the thirteen epidemics discussed above and the one reported by Loosli and associates (8), several other outbreaks of disseminated pneumonitis have occurred. Serologic evidence that the outbreak was histoplasmosis has been obtained in three such epidemics. One, which is being studied by Dr. David Smith and has been referred to by Sabin (3 *b*), involved 7 men who had shoveled pigeon excreta from a church belfry in *Warrenton, North Carolina*, in 1945. Another occurred in 1948 on a farm near *Taneytown, Maryland*, where 4 brothers became ill after shoveling out a cow barn (24). The most recent epidemic (1951) involved 3 workmen who had scraped bat dung from a bridge in Maryland (25).

This type of disease occurs in single cases as well as in epidemics. The writers have studied 2 cases of disseminated pneumonitis diagnosed serologically as histoplasmosis and accompanied by a history of activities similar to those of the epidemics. One case involved a farmer who had recently cleaned out a pig barn, and the other involved a man who had cleaned out a chicken coop. *Histoplasma* was isolated from the chicken coop.

DISCUSSION

With the exception of the infant who died of histoplasmosis associated with the Bellevue, Iowa, epidemic, the fungus was not isolated from patients involved in the series of outbreaks herein reported. The proof of the nature of the etiology of these epidemics is therefore less direct. The nature of the proof presented might well be inconclusive in an individual case. It is the occurrence of groups of cases with similar reactions that makes this type of etiologic evidence secure. Moreover, the series of groups of cases with similar clinical findings and similar reactions to the etiologic studies seemingly provides conclusive evidence of etiology. While a possible criticism of the skin test and serologic evidence might be that a yet unknown fungus closely related antigenically to *Histoplasma capsulatum* could give similar reactions, this is not tenable in the face of the frequent isolation of *Histoplasma* from the point sources of the epidemics.

In the companion paper (9), which presents the epidemiologic studies of these epidemics, it is concluded that the mode of infection in these outbreaks is by inhalation of air-borne *Histoplasma* spores, and that the natural reservoir of the fungus is its saprophytic growth in nature.

SUMMARY

The etiologic studies of a series of epidemics of disseminated pneumonitis are presented.

Of 116 persons involved in the outbreaks, 94 were skin tested and all reacted to histoplasmin.

Complement fixation and precipitin tests for histoplasmosis showed high titer reactions in most of those sera obtained within three months of onset of the epidemics, with falls in titer thereafter. Some serologic evidence of histoplasmosis infection was obtained in each epidemic.

Pulmonary calcification was demonstrated to be present in 61 of 73 persons who were examined roentgenographically at least four years after onset of the epidemic in which they were involved. In 42 of these persons with calcification, the process was the disseminated miliary type.

Histoplasma capsulatum has been cultured from soil samples obtained at the place of infection (point source) in eleven of thirteen epidemics examined.

It is concluded that, because of the occurrence of the disease in groups of people, the reported etiologic studies provide conclusive evidence that the epidemics were histoplasmosis.

SUMARIO

Aparición de Histoplasmosis en Epidemias: Estudios etiológicos

Estos estudios etiológicos comprenden una serie de epidemias de neumonitis diseminada.

De 116 personas que figuraron en los brotes, se hicieron cutirreacciones en 94 y todas reaccionaron a la histoplasmina.

Las pruebas de fijación del complemento y de precipitinas para histoplasmosis acusaron reacciones a títulos altos en la mayor parte de los sueros obtenidos en término de tres meses de la iniciación de las epidemias, con disminución del título a partir de entonces.

Descubrióse calcificación pulmonar en 61 de 73 personas examinadas roentgenográficamente a lo menos cuatro años después de la iniciación de la epidemia en que habían figurado. Cuarenta y dos de las que tenían calcificación revelaban la forma miliar diseminada.

En 11 de 13 epidemias estudiadas se cultivó el *Histoplasma capsulatum* de muestras del terreno obtenidas en el lugar de la infección (punto de concentración).

Dedúcese que, debido a la ocurrencia de la enfermedad en grupos de personas, los estudios etiológicos presentados ofrecen prueba terminante de que las epidemias eran de histoplasmosis.

RESUME

La fréquence de l'histoplasmose dans les épidémies: Etudes étiologiques

Présentation d'une étude étiologique sur une série d'épidémies de pneumonies disséminées.

Parmi les cent seize individus compris dans ces épidémies, un test cutané a été pratiqué chez quatre vingt quatorze sujets. La réaction à l'histoplasmine a été positive chez tous.

Sur un nombre de cent seize individus compris dans ces épidémies, un test cutané a été pratiqué chez quatre vingt quatorze sujets. La réaction à l'histoplasmine a été positive chez tous.

La réaction de fixation du complément et le précipitine-test de l'histoplasmose ont montré des réactions d'un titre très élevé dans la plupart de ceux des sérums qui ont été obtenus dans les trois mois qui ont suivi de début des épidémies; à partir de ce moment, le degré de la réaction s'abaissait.

La calcification pulmonaire a été démontrée chez soixante-et-un sujets sur les soixante-treize pour lesquels des radiographies ont été faites quatre ans au moins après le début de l'épidémie qui les avait affectés. Chez quarante-deux de ces sujets, porteurs de calcification, on observait la forme miliaire disséminée.

Le *Histoplasma capsulatum* a été cultivé à partir d'échantillons du sol prélevés sur les lieux de l'infection ("point source") dans onze épidémies sur les treize ayant été étudiées.

La fréquence de l'histoplasma dans les groupes de la population permet de conclure d'après les études étiologiques rapportées que ces épidémies étaient constituées par l'histoplasmose.

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THE GROWTH OF MAMMALIAN TUBERCLE BACILLI IN TWEEN-ALBUMIN LIQUID MEDIUM[1]

I. Observations on the Proportion of Viable Organisms, the Cytology, and the Virulence of Bacilli at Different Stages of Growth of Strain H37Rv

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INTRODUCTION

The introduction of the Tween-albumin liquid medium (1), which supports dispersed growth of virulent mammalian tubercle bacilli, and the development of relatively accurate methods of performing total and viable counts of tubercle bacilli (2) have made it possible to study the growth of these organisms in liquid culture media in the same manner as with other bacteria. The present paper reports the results of such investigations with a standard laboratory strain of virulent human-type tubercle bacilli, H37Rv, and a succeeding paper describes the results of similar studies with the attenuated bovine strain, BCG.

In addition to total and viable counts of the bacilli at various stages of growth, studies have been made of the bacterial cytology, and measurements made of the bacterial density with a photoelectric absorptiometer. Bloch's demonstration (3) of the different virulence of young and old cultures of mammalian tubercle bacilli suggested the inclusion of an assessment of the virulence of the cultures throughout the growth of the cultures. The effect of the age of the culture on the neutral red test of Dubos and Middlebrook (4) has also been investigated.

MATERIALS AND METHODS

Bacterial strain: Strain H37Rv of *M. tuberculosis* var. *hominis* was kindly provided by Dr. R. J. Dubos.

Culture media: The liquid medium was the Tween-albumin medium (5), containing 0.05 per cent of Tween 80,[2] 0.5 per cent of bovine albumin fraction V, and 0.5 per cent of glucose. Viable counts were carried out on oleic-acid-albumin-agar solid medium poured in three and one-half-inch Petri dishes over a saline-agar base.

Mice: Mice were bred in the Walter and Eliza Hall Institute of Medical Research. The stock, which is multicolored, has been maintained by constant outbreeding between mice of different colors and parentage. Six- and seven-week-old mice of both sexes were used.

Undiluted formalin was added to all samples except those used for the viable counts and animal inoculations so that the final concentration was 1 per cent.

Total bacterial counts: Total bacterial counts were made in a Petroff-Hausser chamber, using a reinforced coverslip and a binocular phase-contrast microscope with oculars ×20 objective ×20 (Zeiss "Lumipan"). A full description of the technique and an assessment of its accuracy has been published (2).

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[2] Tween 80 is the proprietary name of the polyoxyethylene derivative of Sorbitan monooleate, obtainable from the Hill Top Laboratories, Inc., 921 Wm. H. Taft Road, Cincinnati 6, Ohio.

Viable counts: Surface drop counts were made on oleic-acid-albumin-agar medium according to the technique described elsewhere (2).

State of dispersion and proportion of acid-fast to nonacid-fast bacteria: The state of dispersion of the bacilli and the proportion of acid-fast to nonacid-fast organisms was determined on drops of the culture suspension. Two hundred bacterial units (clumps and isolated bacilli) were counted on each occasion. Films of each preparation were prepared daily and saved until the end of the first week, when all were stained by the Ziehl-Neelsen method, care being taken to ensure that the duration of staining and decolorization was the same for each film.

The total number of bacilli per ml. was calculated according to the formula

$$N = n\left(\frac{x}{100}a + \frac{y}{100}b\cdots\right)$$

where N = total number of bacilli per ml.

n = total count (bacterial units per ml.)

$\left(\frac{x}{100}a + \frac{y}{100}b\ldots\right)$ = clumping factor: a, b, et cetera being 1, 2, et cetera bacilli per bacterial unit; x, y, et cetera being the percentage of bacterial units containing a, b, et cetera bacilli per bacterial unit.

Cytology of the bacteria: The cytology was studied with the phase-contrast microscope, using unstained bacilli, and in preparations stained by Robinow's acid-Giemsa method (6). Initial suspension of the bacilli in petrol-ether gave excellent dispersion of the organisms on the coverslip and improved the differentiation between nucleus and cytoplasm. Nuclear counts were made by examining at least 100 individual bacilli, selecting those in which there was a clear differentiation between nucleus and cytoplasm. Examinations were made with a Zeiss "Lumipan" microscope, using an achromatic ×90 oil-immersion objective, 10× oculars, and a green filter.

The length of the bacilli was measured with an ocular micrometer. Films of unstained bacilli, deposited from petrol-ether on a coverslip, fixed with osmium tetroxide, and mounted in water, were examined with the phase-contrast microscope. No attempt was made to measure the thickness of the bacilli.

Turbidity measurements: The optical density was measured in a Hilger-Spekker photoelectric absorptiometer, using neutral glass filters and standardizing against a blank consisting of uninoculated culture medium of the same batch as that used for the cultures. The optical density has been expressed in the tables as the direct scale reading or a multiple thereof.

Preliminary tests with the Spekker absorptiometer indicated that with this instrument, as with other instruments (7, 8), the absorptiometer reading was directly proportional to the concentration of bacilli until relatively concentrated suspensions were used (readings greater than 0.6 with this instrument). In the later stages of growth, cultures were therefore diluted with uninoculated Tween-albumin medium before the determination of the optical density.

Neutral red test: The neutral red test was carried out by the technique described by Dubos and Middlebrook (4).

Virulence for mice: Following Bloch's demonstration of the different virulence for mice and rabbits of cultures of virulent tubercle bacilli of varying ages, tests were made by the intravenous inoculation of 6- to 7-week-old mice with cultures which had been incubated for different periods of time. The cultures were concentrated (or diluted) so that the inoculum contained approximately 5×10^6 bacterial units in the first experiment (*Experiment I*) and approximately 5×10^7 bacterial units per ml. in the second experiment (*Experiment II*).

In *Experiment I* the bacilli were spun out in a horizontal centrifuge, washed in uninoculated Tween-albumin medium, and spun out again. The supernatant fluid was dis-

carded and the deposit resuspended in the volume of uninoculated Tween-albumin medium required to give the estimated concentration. The actual concentration was then checked by making a total count in the Petroff-Hausser chamber. In the second experiment, dilution or concentration was effected without preliminary sedimentation and discard of the supernatant culture medium.

On each occasion 20 mice were inoculated intravenously with 0.1 ml. of the final suspensions. Autopsies were performed on all animals when they died. When it was thought necessary, smears were made from appropriate organs and stained for acid-fast bacilli.

Statistical treatment of results: The statistical handling of the results of the total and viable bacterial counts was discussed in some detail in an earlier publication (2), and similar methods were used here.

The parameters determined were:

$$\text{the mean, } \bar{x} = \frac{\Sigma x}{n}$$

$$\text{the variance, } s^2 = \frac{\Sigma(x - \bar{x})^2}{n - 1}$$

$$\text{the standard error of the mean, } S\bar{x} = \frac{s}{\sqrt{n}}$$

To determine the growth constants, the results obtained in various investigations were converted to $\log_2$ values and plotted on graph paper. The significant portion of the growth curve for the determination of the exponential growth rate was determined by inspection, and a straight line was fitted to the data for this portion of the curve by the method of least squares (9).

EXPERIMENTAL PROCEDURE

Three experiments were performed. The essential differences in their arrangement are shown in table 1. In the first, the inoculum consisted of a 7-day-old culture of H37Rv which had been prepared by inoculating 99 ml. of culture medium with 1 ml. of the original liquid culture prepared from an isolated colony of the organism. This "starter culture" contained 6.8×10^7 bacterial units per ml. Eighty 250 ml. conical flasks, each containing 99 ml. of culture medium, were inoculated with 1.0 ml. of the 7-day-old culture suspension. All culture flasks were brought to 37°C. before inoculation, and the flasks examined at two and four hours after inoculation were incubated in a 37°C. water bath. They were capped with cellophane and placed in an incubator operating at 37°C. Those incubated for more than a week were made up to the original volume by the addition of distilled water if necessary.

At various intervals an appropriate number of flasks were removed. It was impossible to perform all of the tests immediately, and the following routine was adopted. At the time of removal of the culture, total and viable bacterial counts were made, the optical density was measured, and mice were inoculated for virulence tests. Material was also prepared for the other tests. Drops were deposited on slides and stained by the Ziehl-Neelsen method at the end of the first week. The material required for study of the cytology was deposited in the centrifuge and resuspended in petrol-ether. The petrol-ether suspension was stored in the

refrigerator until opportunity offered to make and examine the films stained by Robinow's method. Aliquots of the cultures were stored in the refrigerator, and the neutral red tests on all specimens were carried out together.

Owing to a fault in the preparation of the solid medium, viable counts in the first experiment were irregular and unsatisfactory. A second experiment, similar in general design to the first but with certain modifications, was therefore performed. In *Experiment II* the 7-day-old culture used as the inoculum contained 7.0×10^7 bacterial units per ml. One ml. of this culture was inoculated into 29 ml. of fresh culture, in 100 ml. conical flasks, instead of into 99 ml. of culture as in *Experiment I*. The higher bacterial concentration in the inoculated flasks simplified and rendered more accurate turbidity measurements and total counts made in the early stages of growth. The procedure in the later stages of the experiment were the same as have just been described, but different dosages were used for the inoculation of mice.

TABLE 1

THE TOTAL COUNTS (BACTERIAL UNITS PER ML.) AND ABSORPTIOMETER READINGS OF THE FRESHLY INOCULATED FLASKS

EXPERIMENT NUMBER	AGE OF INOCULUM	RATIO: VOLUME OF INOCULUM / VOLUME OF MEDIUM IN FLASKS	TOTAL COUNT (BACTERIAL UNITS PER ML. $\times 10^6$)	ABSORPTIOMETER READING ($\times$ 1,000)
	days			
I	7	1/99	6.8	—*
II	7	1/29	24.0	15
III A	3	5/25	145.0	44
III B	21	5/30	151.0	29

* Suspension too dilute to register a reading on the absorptiometer.

A further experiment (*Experiment III*) was performed to determine the effect of the age of the inoculum on the growth curves. Two series of flasks were inoculated with the same culture of H37Rv, one when this was three days old (III A) and the other when it was twenty-one days old (III B). The 3-day-old culture was first concentrated by centrifugation, and both were freed of large clumps by centrifugation at a low speed. With the first culture (III A), 5 ml. of inoculum were added to 25 ml. of culture medium in 250 ml. flasks, and the total count of the newly inoculated flask was approximately 1.5×10^7 bacterial units per ml. With the older culture (III B), 5 ml. were inoculated into 30 ml. of medium, and the total count of the newly inoculated flask was also 1.5×10^7 bacterial units per ml. In this experiment each flask yielded three successive samples, instead of separate flasks being used for each sample as in the previous experiments.

RESULTS

The results of the three experiments are condensed in tables 2, 3, and 4. In order to simplify the results, the various investigations are considered separately or in small groups.

Bacterial Growth Phases

The data of *Experiment II* which bear on bacterial growth phases, namely, the total counts, the nephelometric readings, and the estimation of total numbers

TABLE 2

THE RESULTS OF EXPERIMENT I. FLASKS CONTAINING 99 ML. OF MEDIUM WERE INOCULATED WITH 1 ML. OF SEVEN-DAY-OLD CULTURE CONTAINING 6.8 × 10^7 BACTERIAL UNITS PER ML.

AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^6)		CLUMPING* FACTOR	TOTAL BACILLI PER ML. × 10^6 (TOTAL COUNT × CLUMPING FACTOR)	OPTICAL DENSITY (SCALE READING × 1,000)	AVERAGE LENGTH OF BACILLI	AVERAGE NUMBER OF NUCLEI PER BACILLUS	NON-ACID-FAST BACILLI‡	VIRULENCE FOR MICE		
	Mean	Standard Error of Mean							Mean Mortality Times (in Days) at		
									25%§	50%	75%
						μ		per cent			
0 hours	6.8	0.132	4.4	30	—†	2.8	4.3	1.5	34	>60	—
2	7.0	1.280	5.9	41	10	3.0	4.5	0.5			
4	6.8	1.181	6.9	47	12	2.6	4.7	5.0			
6	7.5	1.179	7.1	53	13	3.2	5.0	5.0	52	>75	—
8	7.3	0.583	5.9	43	—	3.4	5.3	10.0			
10	7.3	1.557	6.0	44	—	3.6	5.5	8.0			
12	7.3	1.601	6.2	44	14	3.5	5.6	13.0	68	74	—
14	7.3	0.692	6.4	47	13	3.5	5.6	—			
16	12.8	2.568	4.2	54	—	3.4	4.6	—			
18	12.8	1.917	5.5	70	—	3.5	4.6	8.0	70	85	—
21	12.0	1.572	4.2	50	—	3.5	—	—			
24	20.3	1.766	5.2	106	16	3.4	4.3	6.0	76	>93	—
36	18.3	2.147	6.2	114	14	3.0	—	—			
48	25.3	1.805	5.2	132	21	2.7	3.6	22.0	27	41	57
3 days	34.5	1.659	6.3	217	—	2.4	3.0	9.0	25	27	41
4	90.0	5.627	5.0	450	—	2.4	3.1	1.5	26	30	45
5	317.0	18.6	6.1	1,930	141	2.7	3.5	4.0	43	56	>72
6	337.0	18.8	5.9	1,990	177	2.8	3.8	0.5	25	33	—
7	903.0	34.5	3.8	2,530	253	2.5	3.6	26.0	29	39	—
11	962.0	29.3	4.2	4,040	339	3.0	3.8	10.0	28	56	—
14	1,133.0	98.5	3.4	3,850	450	2.8	3.7	4.0	41	61	—
21	4,075.0	66.8	3.6	14,700	556	2.4	2.6	20.0	41	>70	—

* Clumping factor = average number of bacilli per bacterial unit.

† Material from a number of flasks was unsatisfactory for turbidity determinations, due to the presence of a deposit in the medium.

‡ Figures indicate the percentage of bacterial units containing at least one nonacid-fast tubercle bacillus.

§ = Mean mortality times (days) at times of 25 per cent, 50 per cent, and 75 per cent mortality in the group.

of bacilli, have been converted to $\log_2$ values and are shown graphically in figure 1. More detailed observations, obtained during the first seventy-two hours of growth in cultures initiated with inocula which had been grown for three and twenty-one days (*Experiment III*), have been similarly set out in figure 2. In all instances the figures for optical density have been adjusted so that the initial

TABLE 3

RESULTS OF EXPERIMENT II. FLASKS CONTAINING 20 ML. OF MEDIUM WERE INOCULATED WITH 1 ML. OF SEVEN-DAY-OLD CULTURE CONTAINING 7.0×10^7 BACTERIAL UNITS PER ML.

AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. $\times 10^6$)		VIABLE COUNT (BACTERIAL UNITS PER ML. $\times 10^6$)		RATIO: $\frac{\text{VIABLE COUNT}}{\text{TOTAL COUNT}} \times 100$	CLUMPING FACTOR	TOTAL BACILLI PER ML. $\times 10^6$ (TOTAL COUNT $\times$ CLUMPING FACTOR)	OPTICAL DENSITY (SCALE READING $\times$ 1,000)	AVERAGE LENGTH OF BACILLI	AVERAGE NUMBER OF NUCLEI PER BACILLUS	NONACID-FAST BACILLI	VIRULENCE FOR MICE			
	Mean	Standard Error	Mean	Standard Error								Mean Mortality Times (in Days) at			
												25%	50%	75%	100%
									μ		*per cent*				
0 hours	24	2.004	16	0.748	66	8.9	214	15	3.4	3.4	9	19	21	22	30
4	20	1.510	8	0.470	40			18							
8	25	2.635	21	0.980	84			18							
12	28	4.330	26	1.0774	92	7.3	204	23							
14	22	2.291	16	1.548	73			20							
16	21	2.049	20	1.095	95			20							
20	16	0.936	14	0.794	87			21							
22	25	1.573	16	0.765	64			23	3.6	4.1					
28	40	3.005	—			8.7	348	24			96	20	21	22	30
37½	33	2.945	22	1.502	66	6.5	215	32			93				
48	80	3.893	58	4.908	72	5.1	408	55			93	15	17	19	20
3 days	183	7.516	158	5.321	86	5.2	952	151	2.9	3.5	92	14	16	17	18
4	306	8.965	471	17.62	153	4.3	1,320	280	2.8	3.1	61	19	20	20	23
5	803	19.61	953	12.74	119	3.4	2,730	430			57	21	22	22	24
6	1,290	38.30	1,520	76.45	118	3.9	5,030	600			96	24	26	28	37
7	2,430	90.30	2,180	53.33	89	3.1	7,530	810	2.9	3.0	70	25	27	39	60
9	5,840	224.0	2,700	229.4	46	2.4	14,000	1,200	3.1	3.1	92				
11	9,440	309.7	7,840	619.6	83	2.0	18,900	1,540	3.0	—	52	26	30	48	76
14	14,970	570.0	11,350	421.6	75	1.7	25,400	2,000	3.0	3.9	5	43	83	110	119
21	19,050	441.5	15,330	631.8	80	2.5	47,600	2,860	2.8	2.9	50	87	100	109	128
28	15,580	514.3	17,530	537.2	113	2.2	33,200	2,930	2.6	3.4	32	45	90	109	129

Symbols as in table 2.

ordinates for total count (bacterial units) and the absorptiometer reading were the same.

Straight lines were fitted by the method of least squares to all data in the interval during which the rate of increase appeared to be exponential (usually twenty-four to seventy-two hours, inclusive) and the following growth constants (10) were determined from regression equations (R and G) or the graphs (T_1 and L).

$$\text{Exponential growth rate } (R) = \frac{\log_2 x_2 - \log_2 x_1}{t_2 - t_1}$$

$$\text{Generation time } (G) = \frac{1}{R} \text{ hours}$$

$$\text{Lag time } (T_1) = t_r - \frac{\log_2 x_r - \log_2 x_o}{R}$$

$$\text{Growth lag } (L) = T_1 . R.$$

x_o, x_r, x_1, x_2 are the optical densities (or bacterial counts) at times o, r, t_1, and t_2.

The results obtained are set out in table 5.

The exponential growth phase: In *Experiment II* the optical density of the cultures increased exponentially between the end of the first day and the end of the third day. Thereafter there was an increasing retardation of growth, as determined by turbidity measurements. Exponential increase in the number of bacterial units persisted for a day longer, until the end of the fourth day, and the subsequent retardation was slower than that of the optical density. This divergence could not be completely explained by a decrease in the degree of aggregation of the bacilli, although there was a definite decrease in the average number of bacilli in each bacterial unit. The other contributing factor was probably the decrease in size of the individual bacilli with the passage of time.

The results obtained in *Experiment I* were on the whole in agreement with those recorded in *Experiment II*, although the low concentration of bacilli in the inoculum rendered accurate total counts and turbidity measurements difficult to obtain during the first two days of the experiment.

The more accurate measurements of *Experiment III*, obtained by the use of larger inocula and more frequent sampling, allowed more accurate calculations to be made of the exponential growth rate and the generation time. With the 3-day-old (III A) and 21-day-old (III B) inocula, the slopes of the curves were almost identical. The generation time during the period of exponential growth was approximately eighteen hours.

The generation times determined from total counts (bacterial units) are also shown in table 5. In the experiments with large 3-day-old and 21-day-old inocula, the values were almost identical with those obtained by consideration of the optical density, but in *Experiment II* the generation time based on bacterial counts was longer than that determined from the optical density of the cultures.

The lag phase: It is not possible to differentiate accurately the lag and acceleration phases in any of the curves obtained, but consideration of figure 2 (*Experiment III*), in which estimations of the opacity and the number of bacterial units were made at frequent intervals during the first forty-eight hours, suggests that there was little detectable change in either for approximately four hours. Both the

TABLE 4

RESULTS OF EXPERIMENT III. FLASKS CONTAINING 25 ML. (III A) OR 30 ML. (III B) OF MEDIUM WERE INOCULATED WITH 5 ML. OF CULTURE OF AGES SHOWN

AGE OF INOCULUM	AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^6)		VIABLE COUNT (BACTERIAL UNITS PER ML. × 10^6)		CLUMPING FACTOR	TOTAL BACILLI PER ML. × 10^6 (TOTAL COUNT × CLUMPING FACTOR)	OPTICAL DENSITY (SCALE READING × 1,000)	AVERAGE LENGTH OF BACILLI	AVERAGE NUMBER OF NUCLEI PER BACILLUS	PERCENTAGE OF BACTERIAL UNITS CONTAINING AT LEAST ONE NON-ACID-FAST BACILLUS
		Mean	Standard Error	Mean	Standard Error				μ		
	0 hours	145	7.325	88	3.352	2.7	392	44	3.1		2.5
	2	149	9.00	103	8.08	2.5	373	45	3.7		3.5
	4	157	6.45			2.6	408	45	3.5		4.5
	7	152	5.69			2.7	410	53	—		15.5
	9	171	4.52			2.3	393	55	4.0		7.0
3 days	14	178	7.86			2.9	516	57	—		13.5
III A	16	195	10.83			2.9	566	—	3.6		11.5
	18	258	11.13			2.5	645	64	—		1.5
	22	256	9.82			2.7	691	85	3.0		1.5
	26	328	15.35			3.5	1,150	97	—		19.0
	39	384	14.01			3.3	1,270	145	—		7.0
	49	869	48.0			3.7	3,220	224	2.4		2.5
	0	151	5.98	125	4.872	2.6	393	29	2.5		6.0
	2	134	7.84	102	—	2.7	361	29	2.4		32.5
	4	160	8.81			2.5	400	29	2.7		5.0
	8	185	9.13			2.2	407	34	3.7		6.0
	12	173	10.76			2.7	467	37	3.2		7.0
21 days	16	199	10.43			—		43	3.0		—
III B	20	193	9.3			2.2	425	45	3.3		7.0
	24	248	10.7			—		52	3.3		—
	28	293	11.53			2.3	674	61			7.0
	36	394	15.99			2.7	1,064	80			8.0
	49	636	18.7			2.2	1,400	137	2.5		12.0
	72	1,360	46.36			2.6	3,540	293	2.1		1.0

Symbols as in table 2.

lag and acceleration phases are included in the lag time (T_1). From table 5 it appears that the lag time was only slightly shorter when a 3-day-old culture was used to initiate the culture than when a 21-day-old culture was used. The lag time was the same with the 3-day-old inoculum (III A), whether determined by measurements of the optical density or the total count, and with the 21-day-old inoculum (III B) the interval was slightly longer when determined by the

results of the total counts. Microscopic observation of the bacterial cells, the results of which are described at some length in a later section, showed that ob-

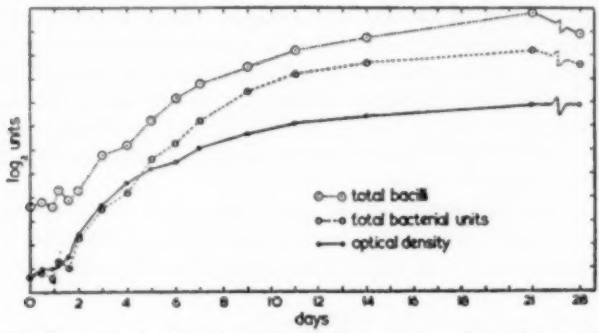

FIG. 1. Growth curves in *Experiment II*. Ordinates: $\log_2$ values of the optical density, the total count of bacterial units per ml., and the total bacilli per ml. of cultures incubated for periods varying from 12 hours to 28 days. Values for optical density adjusted so that the initial value coincides with that of the total count (bacterial units). Total bacilli per ml. equals total bacterial units per ml. (counted in Petroff-Hausser chamber) × clumping factor (average number of bacilli per bacterial unit).

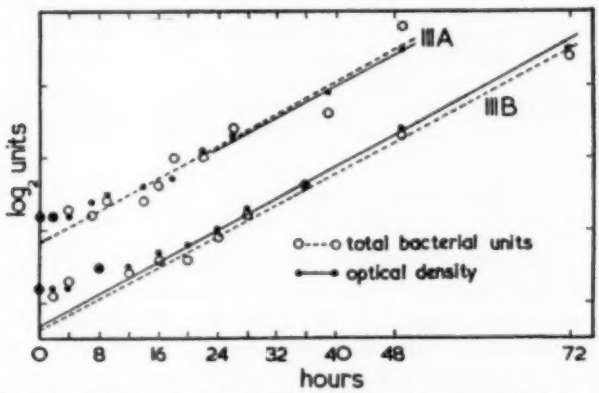

FIG. 2. Growth curves in *Experiment III*. Ordinates: $\log_2$ values of the optical density and the total count of bacterial units per ml. (counted in Petroff-Hausser chamber) of cultures incubated for periods varying from 2 to 72 hours. Values for optical density adjusted so that the initial values coincide with those of the total counts. Straight lines fitted by the method of least squares for the exponential growth phase. III A, 3-day-old inoculum; III B, 21-day-old inoculum.

servable changes in the size of the cells occurred within two hours of inoculation in III A, but not until the fourth hour in III B.

The short lag periods which occurred when cultures grown for shorter or longer periods in the Tween-albumin liquid medium were reinoculated into

fresh batches of the same medium are in striking contrast to the results recorded by Engbaek (11), who compared, by direct microscopic observation, the initial growth on oleic-acid–albumin–agar solid medium of cultures which had been grown for three days and two or three weeks in Tween-albumin medium. The young cultures grew uniformly and without any noticeable latent period, whereas with the 3-week-old cultures there was a distinct latent period, and the bacteria appeared to be less refractive and more granulated. The size of the colonies was very much less after four days' cultivation of the old bacilli than of the young bacilli, a result which he considered was not completely explained by the greater clumping of the younger cultures.

Viable counts of the contents of the newly inoculated flasks (tables 3 and 4) showed that the observed lag was a true physiologic process, and not an artefact due to the inclusion of a large proportion of nonviable cells in the inoculum (12).

TABLE 5

GROWTH CONSTANTS OF H37Rv GROWN IN TWEEN-ALBUMIN MEDIUM

EXPERIMENT	PROPERTY MEASURED	INTERVALS	EXPONENTIAL GROWTH RATE (R)	GENERATION TIME ($1/R$)	LAG TIME (T_l)	GROWTH LAG (L)
		hours		*hours*	*hours*	
II	Opacity	28–72	0.061	16.0	21.0	1.0
	Total bacilli	48–144	0.045	22.0	40.0	1.47
	Total bacterial units	28–120	0.044	23.0	11.0	0.45
III A (3-day-old inoculum)	Opacity	14–49	0.053	18.8	7.0	0.37
	Total bacterial units	16–49	0.056	17.8	7.0	0.37
III B (21-day-old inoculum)	Opacity	24–72	0.054	18.5	9.6	0.52
	Total bacterial units	24–72	0.054	18.5	10.5	0.56

The phase of retardation: With tubercle bacilli growing in a dispersed state in the Tween-albumin medium, arithmetic linear growth occurs after the end of the exponential growth phase (13, 14). Fisher and Kirchheimer observed the occurrence of this stage of arithmetic linear growth by plotting the readings for the optical density arithmetically instead of logarithmically. In figure 3 the total counts and absorptiometer readings obtained in *Experiment II* have been plotted on an arithmetic scale and clearly indicate an arithmetic linear increase in the opacity between the third or fourth day, when the phase of exponential growth ceases, and approximately the seventeenth day. The arithmetic linear increase in the count of bacterial units was less prolonged (seven to fourteen days). Examination of figure 2 shows that these periods coincide fairly accurately with the phase of retardation of these two properties of the culture.

Stationary phase: The optical density remained unchanged between the twenty-first and twenty-eighth days of *Experiment II*, but there was a slight fall (20 per cent) in the cell count. There was no evidence of autolysis similar to that

reported to occur by Schaefer, Marshak, and Burkhart (8) when they used culture medium containing an excess of glucose and limiting amounts of a nitrogen source.

The yield of bacilli: The yield of bacilli, in terms of bacterial mass (optical density) and numbers of bacilli, at weekly intervals after inoculation is shown in table 6. *Experiment III* was terminated too soon to determine these values. The yield in the second experiment, in which the inoculum was larger and the volume

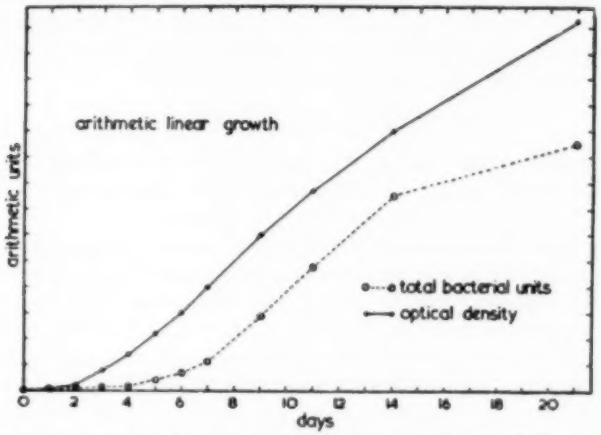

FIG. 3. Growth curves in *Experiment II* plotted arithmetically. Ordinates: values for optical density and total bacterial units per ml. (counted in Petroff-Hausser chamber) plotted on an arithmetic scale.

TABLE 6

THE YIELD AT VARIOUS INTERVALS AFTER INOCULATION, EXPRESSED AS TOTAL COUNT (BACTERIAL UNITS PER ML.), TOTAL BACILLI, AND OPTICAL DENSITY

EXPERIMENT	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^7)			TOTAL BACILLI PER ML. × 10^7			OPTICAL DENSITY (SCALE READING × 1,000)		
	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
I	9.1	11.3	40.1	25	38	147	253	450	556
II	24.3	150.0	190.0	75	254	476	810	2,000	2,860

of fluid in the culture flask smaller, was considerably higher than in the first experiment and was of the same order as the yields of BCG obtained under similar conditions (*see* paper II, which follows).

The relationship of the viable and total counts: Wilson and Schwabacher (16) reported that there were only 5 to 8 per cent of living bacilli in a suspension of mammalian tubercle bacilli prepared by grinding with a mortar and pestle the growth obtained from the surface of an egg medium. Owing to the unsuitability of the media used for the detection of viable organisms, they considered that this

figure might be an underestimation of the true position, but Wilson believed that, with tubercle bacilli, as with other microorganisms (17), only a proportion (and probably a small proportion) of bacilli in a culture were viable and capable of reproduction. Experiments reported earlier (2) indicated that, when a variety of pathogenic *Mycobacteria* were grown in Tween-albumin medium and tested for their viability on oleic-acid-albumin-agar medium, there was almost always a close correspondence between the total count and the viable count. The results shown in table 3 confirm this view and indicate that the same high ratio of viable to total bacterial units existed throughout the twenty-eight days of the experiment. This conclusion is confirmed by the direct microscopic observations reported by Espersen (18) and Engbaek (11).

Cytologic Observations

The morphology of the bacteria was examined from several aspects, namely, the size of the bacilli, their state of aggregation, the numbers of nuclei, and the appearance after staining by the Ziehl-Neelsen technique.

Size of the bacilli: The size of the bacterial cells varied considerably at different stages of growth. The length measurements of randomly selected bacteria from cultures of various ages (*Experiment III*) are shown graphically in figures 4 and 5. Average lengths are shown in the appropriate tables. The composition of the "starter" cultures, in terms of the sizes of the bacterial cells, varied according to the age of the culture. In the 3-day-old "starter" culture, there were a considerable number of elongated cells which were not present in the older "starter" cultures. With the 3-day-old "starter" culture, there was a pronounced shift to the right in the histogram, i.e., a larger proportion of elongated bacilli, by the end of the second hour after cultivation, whereas the change at this time in the 21-day-old culture was slight. In the latter culture also, however, there were no very small bacilli present in the four-hour specimen, and there was then an increasing number of longer forms. In both cultures the maximum average length of bacilli and the greatest number of long organisms occurred eight hours after inoculation. Thereafter the number of long forms diminished until, by the forty-eighth hour, there were very few long bacilli and, by the end of the third day, the average length of the bacilli was slightly more than half that found at the eighth hour. These changes can readily be seen in figure 5, in which the average length of the bacilli and the percentages of small (less than 2.5μ) and large (more than 4μ) bacilli are plotted.

In both experiments the average size of the bacilli at the end of the third day was much less than in the original "starter" cultures, even the 21-day-old one. This relatively rapid transition of the bacilli to a mature morphology was doubtless due to the size of the initial inoculum in these experiments. They were more than twenty times as concentrated as the freshly inoculated cultures used for preparation of the "starter" cultures. It is possible, also, that on more prolonged incubation an increase in cell length might have occurred at about the sixth day.

The frequency of short (less than 2μ) and long (more than 3μ) bacterial cells,

together with the average length in microns of the same cells as those on which the nuclear counts were made, are set out in figure 6 (top). The "starter" culture in this experiment was a 7-day-old culture. There was no significant change in the length of the bacilli at the two-hour and four-hour counts, but the proportion of longer bacilli was greatly increased at six hours. Thereafter, the proportion of long bacilli and the average length of the bacilli rose to reach a maximum at the tenth hour and continued at a high level until the twenty-first hour. After the

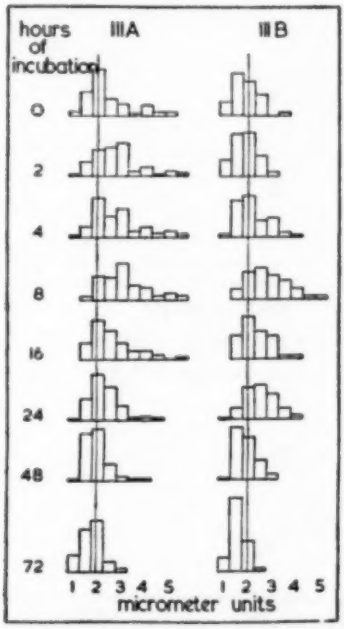

FIG. 4. Histograms showing the distribution of bacilli of different lengths at various intervals after inoculation of the culture medium in *Experiment III*. III A, inoculum consists of 3-day-old culture; III B, inoculum consists of 21-day-old culture. Distributions shown as the percentage of bacilli measuring 1, (1.5), 2, et cetera micrometer units (1 micrometer unit = 1.3μ).

end of the first day, the proportion of smaller bacilli rose appreciably and fluctuated between 20 and 30 per cent for the remainder of the experiment. There was an increase in the proportion of longer bacilli between the fifth and the fourteenth days, and the average length of the bacilli rose during this period also.

There was no evidence in these results that any of the bacilli corresponded to the "B" forms of Pulvertaft (19) and failed to proliferate. At no stage either during the performance of the total counts or the examination of the Giemsa-stained smears were any abnormally small objects seen which might correspond to the filterable forms of tubercle bacilli described by some writers, nor was there

a disproportion between total and viable counts which might be ascribed to small forms unrecognized in the total counts but capable of initiating the development of a colony on the solid medium.

The nuclear apparatus: Nuclear staining of tubercle bacilli presents certain technical difficulties, and the preparations were by no means as clear as those made with other species of bacteria. Nevertheless, in the majority of stained preparations, dark areas which were interpreted as nuclei were seen with about

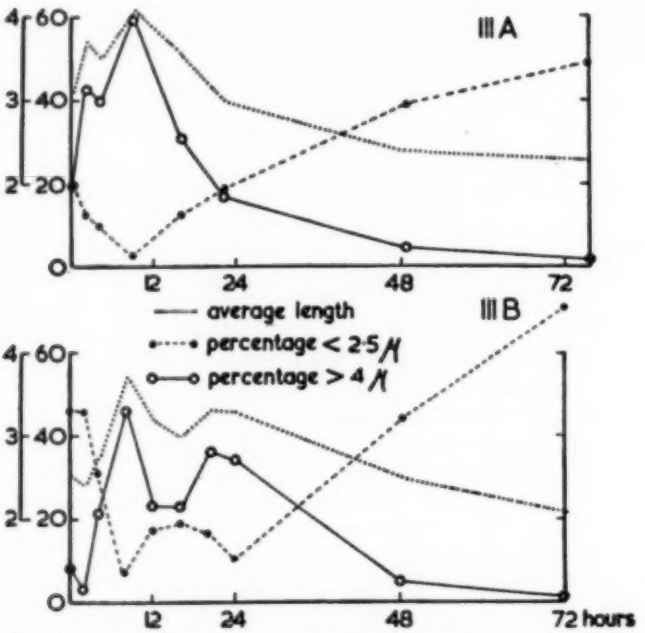

FIG. 5. The changes in the average length of bacilli, and the percentages of small (less than 2.5μ) and large (more than 4μ) bacilli at various intervals after inoculation of the culture flasks in *Experiment III*. III A, inoculum consists of 3-day-old culture; III B, inoculum consists of 21-day-old culture. Ordinates: 2, 3, 4 = average length of bacilli (μ); 20, 40, et cetera = percentages of short and long bacilli.

the same degree of clarity as they are shown in the photographs in Bisset's paper (20). Direct examination of fixed unstained bacilli by phase contrast suggested that the dark areas of the stained bacilli corresponded to areas of similar size and location in the unstained preparations, as Tulasne (21) and Stempen (22) found with *E. coli* and *Proteus vulgaris*.

The relative proportions of bacilli with few (one or two) nuclei and many (five or more) nuclei, together with the average number of nuclei per bacterium, in cultures of different ages in *Experiment I* are shown graphically in figure 6 (bottom). Figures for the average number of nuclei per cell are shown in table 1.

An increase in the proportion of cells with several nuclei occurred within two hours of inoculation of the culture flasks, even though there was at this time no increase in the length of the bacilli. The number of cells with few nuclei fell greatly, and those with many nuclei increased greatly, up to the fourteenth hour after inoculation. The average number of nuclei per bacillus then fell rapidly, this fall preceding by some ten hours the decrease in length of the bacilli (figure 6). After dropping to a low value at the end of the third day, the average number of nuclei per bacterium rose somewhat and remained fairly constant until the fourteenth day. In old cultures (21 days and 28 days) the bulk of the

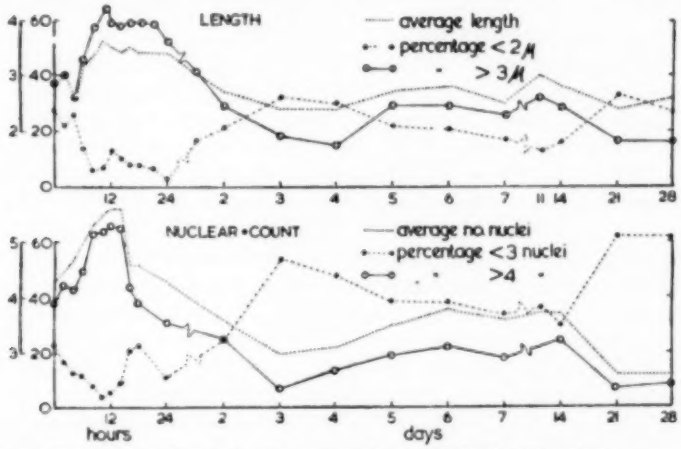

FIG. 6. (Upper) The changes in the average length of the bacilli and the percentages of small (less than 2μ) and large (more than 3μ) bacilli at various intervals after inoculation of the culture flasks in *Experiment I* (inoculum consists of 7-day-old culture). Ordinates as in figure 5.

(Lower) The changes in the average numbers of nuclei per bacillus and the percentages of bacilli with few (less than 3) or many (more than 4) nuclei at various intervals after inoculation of the culture flasks in *Experiment I*. Ordinates: 3, 4, 5 = average number of nuclei per bacillus; 20, 40, et cetera = percentages.

cells contained only a single nucleus (approximately 15 per cent) or two nuclei (approximately 45 per cent).

In *Experiment II*, nuclear counts were made only at daily intervals. The general trend of the results was much as in *Experiment I* but, as the crucial early counts were not carried out, detailed discussion is not necessary.

The majority of the cells of H37RV are multinucleate at all stages of the life cycle. During the period between (and including) the eighth to the fourteenth hours after inoculation, there are in the culture a large number of elongated bacilli with many nuclei; and a few extreme forms occur. It is possible that the steady position of the nuclear count between the fifth and fourteenth days, at a level higher than found either just before or afterwards, is related to the arithmetic linear growth which occurs during this phase of the growth cycle.

Aggregation of the bacilli: Several investigators (3, 24) have noted that, in very young cultures of mammalian tubercle bacilli in the Tween-albumin medium, the microorganisms are somewhat more aggregated than in older cultures. In the experiments reported here the degree of aggregation was determined by the examination of suitably diluted drops of culture suspensions, which were stained by the Ziehl-Neelsen method. The numbers of bacilli per bacterial clump were counted and recorded as 1, 2, 3, 4, 5, approximately 7, 10, 20, 30, 40, 50. One or two hundred bacterial units were examined for each preparation. The results are shown in tables 1–3 as an average number of bacilli per clump, the clumping factor. In *Experiment III*, in which the starter cultures were centrifuged

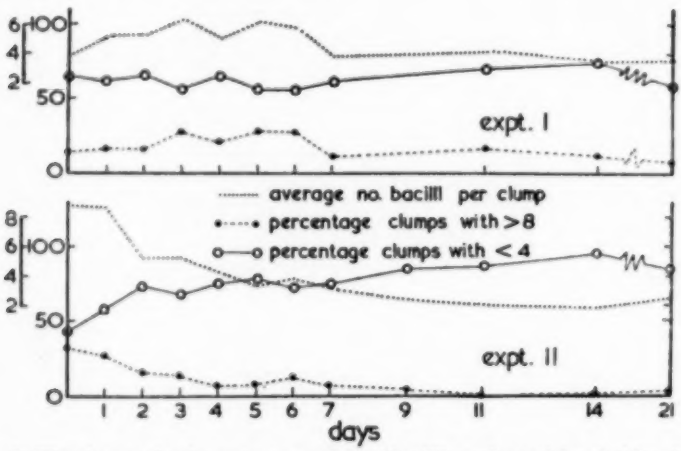

FIG. 7. The changes in the average number of bacilli per bacterial unit (the clumping factor) and the percentages of units containing few (1, 2, or 3) bacilli and those containing many (more than 8) bacilli, at various intervals after inoculation of the culture flasks in *Experiment I* (upper figure) and *Experiment II* (lower figure). Ordinates: 2, 4, 6, et cetera = average numbers of bacilli per bacterial unit; 50, 100 = percentages of dispersed and clumped bacilli.

to remove the larger clumps, there was a relatively slight increase in the degree of aggregation during the three days of the experiment. More useful for the determination of the changes in aggregation with the age of the culture are the results obtained in *Experiments I* and *II*. These are shown graphically in figure 7. In *Experiment I* the clumping factor and the percentage of clumps containing 10 or more bacilli were maximal between the second and the sixth days. After the end of the first week there was a steady rise in the proportion of isolated bacilli and those in small groups (three or less) and a steady fall in the clumping factor. In *Experiment II* the initial suspension contained a relatively large number of fairly large clumps of bacilli, so that the clumping factor was initially quite high. It fell continuously throughout the experiment. Between the second and the sixth days the clumping factor and proportion of larger clumps were

relatively static, but after the end of the first week the number of small clumps and isolated bacilli increased greatly. The high initial values recorded in *Experiment II* were due to failure to remove the larger clumps from the inoculum by light centrifugation. These larger clumps probably developed to this degree in the "starter" cultures but not in the growth experiments because in the latter the flasks were shaken daily, whereas "starter" cultures were not agitated until preparation of the inoculum. If allowance is made for this complicating factor, the present results are in confirmation of the suggestions of other writers. Fourteen- and twenty-one-day-old cultures appeared to be much better dispersed than cultures less than a week old.

Degree of acid-fastness: Negré (23) has published a number of papers concerned with the nonacid-fast bacilli observed in young cultures of virulent tubercle bacilli and the relative avirulence of these cultures. His studies were made with bacilli grown on Sauton's medium, and he found the diminished virulence and lack of acid-fastness most pronounced in cultures three to four days old. Bloch (3) pointed out that, with Tween-albumin cultures, much the same features were observed with one-day-old cultures as Negré described with 4-day-old Sauton cultures.

In the present experiments the numbers of bacterial units which contained at least one nonacid-fast bacillus were noted during the determinations of the degree of clumping. The results were extremely variable, both from one specimen to another, withdrawn a few hours later, and between the different experiments. This is apparent from the relevant columns of tables 1, 2, and 3. The percentage of nonacid-fast tubercle bacilli in the smears was always a good deal less than the figures shown in the tables might suggest, for often there was only one nonacid-fast bacillus in a clump containing 10 or more acid-fast bacilli. The present results with H37Rv would not permit any generalizations upon possible relationships between acid-fastness and age of the culture under the conditions of the experiments.

Virulence for Mice

Bloch's (3) observation that 3-day-old Tween-albumin cultures of tubercle bacilli are more virulent for mice than 3-week-old cultures has been confirmed (24). An attempt was made to determine at what stage of the growth cycle the observed changes in virulence occurred and to see whether they bore any relation to the stage of the growth cycle or any other factor. In *Experiment I*, mice were inoculated with 5×10^5 bacterial units of washed bacilli, and with a tenfold dilution of this. None of the mice which received the smaller inoculum died in the first 100 days, and they will not be considered further. Although this inoculum was of the same order as that employed by Bloch (2×10^5 with young cultures; 12×10^5 with old cultures), the mice did not succumb as uniformly and rapidly as did those of Bloch. The usual responses resembled those shown by CF1 mice inoculated with smaller doses of H37Rv (25), or Tumblebrook mice inoculated with the Ravenel strain (26). Plots on probability paper did not show a linear relationship, and the time for 50 per cent mortality could not be used to express

the relative virulence of the various cultures inoculated. Instead, the practice of Stewart and Tamargo-Sanchez (24) was followed and the average survival time of the first 25 per cent, the first 50 per cent, and the first 75 per cent of mice to die was recorded (table 1). In spite of the survival of a considerable number of mice throughout the experiment, the results show an unmistakable trend in the virulence of the cultures. Within six hours of the initiation of the new culture, the virulence of the bacilli had dropped appreciably, and this decline in virulence continued until the minimum virulence was shown by the 24-hour-old culture. At forty-eight hours, however, when the growth had entered the exponential phase, the virulence increased sharply and reached a maximum figure on the third day. Thereafter it diminished slowly and steadily to reach a relatively low figure at twenty-one days.

In *Experiment II*, in an attempt to kill within a reasonable period all mice inoculated with the more virulent cultures, the dose was increased approximately tenfold, and the bacilli were not washed but were inoculated in the medium (appropriately concentrated or diluted) in which they had been grown. The desired result was achieved, but in the process the diminished virulence of the one-day-old culture was obscured. Thereafter, however, the trend was the same as in *Experiment I*. The 2- and 3-day-old cultures showed maximum virulence, which then diminished progressively until it had fallen to a relatively low level by the seventh day and a very low level by the twenty-first day.

Considering the results of both experiments together, there appears to be a fairly close correlation between the stage of the growth cycle and the virulence of the bacilli. During the first twenty-four hours, which in these experiments, it will be remembered, was almost completely occupied by the lag and acceleration phases, the virulence was low. Then, with the onset of the exponential phase, virulence rose rapidly to a high level and declined steadily when growth passed through the exponential phase into the phase of retardation. It is possible that the pronounced changes in virulence to which Bloch directed attention are due, not to variations in the content of "cord factor," but to the stage of the growth phase of the inoculated bacilli; or, on the other hand, the production of "cord factor" may well vary at different phases of the growth cycle.

None of these mice exhibited at death the pathologic picture of acute septicemia described by Bloch. However, all of those which died within the first thirty days had focal lesions in the myocardium, sometimes very numerous. This difference is probably related either to the resistance of the strain of mice used or, perhaps, to the virulence of the substrain of H37Rv employed in our experiments.

The Neutral Red Test

The neutral red test (4) was performed on the deposit obtained by centrifugation of an aliquot of the culture at various stages of each of the experiments just described. As far as could be determined, with the very small amounts of bacilli present in the early stages of growth of most of these experiments, the result was positive throughout, the bacilli taking on a reddish coloration. However, in view of the report by Viallier and Tigaud (27) that the young bacilli of virulent

strains growing at the periphery of colonies in Youmans' medium remain yellow, compared with the older red bacilli, at the center of the colonies, the problem was reinvestigated. A liquid culture medium containing no Tween 80 was inoculated with a large amount of a 7-day-old Tween-albumin culture. The sediment obtained by centrifugation was examined at frequent intervals. The color of the deposit was compared with the colors of the Hendon Johnson & Sons Universal Indicator Papers. In table 7 the number indicates the pH color value with which the deposit could be most accurately matched. It is apparent that, although the bacilli never failed to take up the dye, the intensity of the red coloration was distinctly lower than usual from the eighteenth to the forty-eighth hours.

The observations presented above are considered under "Discussion" in the paper which immediately follows the present one.

TABLE 7

THE NEUTRAL RED TEST

Numbers Indicate the Best Match with the Colors on the Universal Indicator Papers (1 = color at pH 1 et cetera)

	AGE OF CULTURE (HOURS)							
	0	6	12	18	24	36	48	72
Color value	1	1	1	2	2	2	1–2	1

SUMMARY

The growth of *M. tuberculosis* (H37Rv) in Tween-albumin liquid medium was studied by a variety of techniques, with frequent sampling during the first forty-eight hours after seeding and observations at longer intervals thereafter for a total period of one month.

When 1 ml. of a 7-day-old culture was inoculated into 99 ml. of culture medium, the first change observed was an increase in the average number of nuclei[3] per bacillus. This was evident two hours after inoculation, and by the fourth hour the bacilli commenced to increase in length. During the next twenty-four hours they became considerably elongated, and the majority of organisms appeared to have five or more nuclei. During the second day the length and nuclear number fell again. Between the fifth and the fourteenth days the bacilli became somewhat longer again; and the average size and number of nuclei per bacillus fell to low levels on the twenty-first day.

The optical density of the suspensions began to rise between six and twenty-four hours after inoculation of the flask, the interval being shorter with the larger inocula and slightly shorter with the 3-day-old inoculum than with the 21-day-old inoculum. The bacterial count increased shortly afterwards. After a short

[3] The dark-staining areas seen when tubercle bacilli which have been subjected to the correct degree of acid hydrolysis are stained by Giemsa are regarded as bacterial nuclei (*see* text).

period of acceleration, the increase in both properties became exponential, and the generation time of H37Rv during this period was approximately eighteen hours. The exponential phase was followed by the retardation phase, during which arithmetic linear growth occurred. From approximately the fourteenth day the cell population and optical density remained stationary. During the first few days the degree of aggregation increased somewhat and then remained constant for approximately a week. Thereafter the bacilli became more completely dispersed.

The ratio of the viable count to the total count fluctuated somewhat but it was never far from 100 per cent.

The neutral red test gave a strongly positive reaction throughout except for the period from the eighteenth to the forty-eighth hours, when the color reaction was orange rather than red.

The virulence for mice, tested by intravenous inoculation, fell to a relatively low value during the first twenty-four hours, rose to a high level on the third and fourth days, and then gradually fell to a low level at the end of the third week.

The proportion of nonacid-fast bacilli fluctuated considerably, without any discernible relationship to the age of the culture.

SUMARIO

El Desarrollo de los Bacilos Tuberculosos de Mamífero en Medio Líquido de Tween-Albúmina: I. Observaciones sobre la Proporción de Microbios Viables, la Citología y la Virulencia de los Bacilos en Diversos Períodos de Desarrollo de la Cepa H37Rv

El desarrollo de H37Rv en el medio líquido de Tween-albúmina fué estudiado por medio de la obtención de muestras frecuentemente durante las primeras cuarenta y ocho horas y a plazos más largos durante un mes.

Al inocular 1 ml. de un cultivo de siete días en 99 ml. de medio de cultivo, la primera modificación observada consistió en un aumento del promedio de núcleos por bacilo. Esto ya era evidente a las dos horas de la inoculación y para la cuarta hora los bacilos comenzaron a aumentar de largo. Durante las próximas veinticuatro horas se alargaron considerablemente y parecía que la mayoría de los gérmenes tenían cinco o más núcleos. Durante el segundo día el largo y el número de núcleos descendieron. Entre el quinto y el décimocuarto días los bacilos se alargaron de nuevo algo y luego el tamaño y el número de nucleos por bacilo bajaron de nuevo, por término medio, al vigésimoprimer día.

La densidad óptica de las suspensiones comenzó a elevarse entre seis y veinticuatro horas después de la inoculación del balón, volviéndose el plazo más breve con los inóculos más grandes y un poco más corto con el inóculo de tres días que con el de veintiún días. La numeración bacteriana aumentó poco después. Tras un breve período de aceleración, el aumento de ambas propiedades se volvió exponencial, siendo de unas dieciocho horas el tiempo de generación de H37Rv durante ese período. La fase exponencial fué seguida de la fase de retardo, durante la cual el desarrollo tomó forma aritmética lineal. A partir más o menos del décimocuarto día la población celular y la densidad óptica permanecieron estacionarias. Durante los primeros días aumentó algo la intensidad de la agregación, para permanecer después constante aproximadamente durante una semana. A partir de entonces, los bacilos se dispersaron más completamente.

La proporción de la numeración viable a la numeración total fluctuó algo, pero jamás se apartó mucho de 100 por ciento.

La prueba del rojo neutro arrojó una reacción fuertemente positiva durante todo el tiempo, excepto por el período comprendido desde la décimaoctava a la cuadrágesimaoctava horas, en que la reacción crómica fué anaranjada más bien que roja.

La virulencia para los ratones, determinada por medio de la inoculación endovenosa, descendió a una cifra relativamente baja durante las primeras veinticuatro horas, se elevó mucho al tercer y cuarto días y luego descendió gradualmente a una cifra baja al final de la tercera semana.

La proporción de bacilos no ácidorresistentes fluctuó considerablemente, sin ninguna relación discernible con la edad del cultivo.

RESUME

La croissance des bacilles tuberculeux de mammifères dans un milieu liquide Tween-albumine: I. Observations sur la proportion d'organismes viables, la cytologie et la virulence des bacilles à différents stades de la croissance dans la souche H37Rv

La croissance de H37Rv dans le milieu liquide Tween-albumine, fut étudié sur des échantillons prélevés fréquemment au cours des premières quarante-huit heures et par des prélèvements à intervalles plus longs pendant un mois.

Quand 1 ml. d'une culture âgé de sept jours était repiqué dans 99 ml. du milieu de culture, le premier changement observé était une augmentation du nombre moyen des noyaux par bacille. Ceci était manifeste deux heures après l'ensemencement et, vers la quatrième heure, les bacilles commençaient à augmenter de longueur. Au cours des vingt-quatre heures suivantes, ils s'allongeaient considérablement et la plupart des organismes semblaient avoir quatre noyaux ou plus. Au cours de la deuxième journée, la longueur du bacille et le nombre des noyaux diminuaient. Entre le cinquième et le quatorzième jours, les bacilles s'allongaient de nouveau dans une certaine mesure; puis, au vingt-et-unième jour, la taille moyenne et le nombre des noyaux par bacille retournaient à un niveau bas.

La densité optique des suspensions commençait à s'accroître entre la sixième et la vingt-quatrième heure succédant à l'ensemencement du ballon, l'intervalle devenant plus court quand l'inoculum était plus gros; cet intervalle devenait également plus court avec l'inoculum de trois jours qu'avec celui âgé de vingt-et-un jours. Le nombre des bactéries augmentait bientôt après. Succédant à une courte période d'accélération l'augmentation de ces deux propriétés devenait exponentielle, et le temps de reproduction pour la souche H37Rv pendant cette période était approximativement de dix-huit heures. La phase exponentielle était suivie par une phase de retardement pendant laquelle un accroissement linéaire arithmétique se produisait. A partir du quatorzième jour, la population cellulaire et la densité optique demeuraient stationnaires. Au cours des quelques premiers jours, le degré de l'agrégation était en progression légère; il demeurait ensuite constant pendant environ une semaine. A partir de ce moment, la dispersion des bacilles devenait plus complète.

La proportion du nombre viable par rapport au nombre total fluctuait un peu, mais elle n'était jamais loin d'atteindre 100 pour cent.

Le test du rouge-neutre donnait une réaction fortement positive pendant toute la durée de l'expérience, sauf pendant la période comprise entre la dix-huitième et la quarante-huitième heure, où la coloration était plutôt orange que rouge.

Chez les souris, soumises au test de l'inoculation intraveineuse, la virulence tombait à un niveau relativement bas au cours des premières vingt quatre heures, elle atteignait un niveau élevé le troisième et le quatrième jours et tombait graduellement à un niveau bas à la fin de la troisième semaine.

La proportion des bacilles non-acido-résistants fluctuait, sans qu'on puisse discerner un rapport quelconque avec l'âge de la culture.

REFERENCES

The references for the present paper are listed at the end of the succeeding paper.

THE GROWTH OF MAMMALIAN TUBERCLE BACILLI IN TWEEN-ALBUMIN LIQUID MEDIUM[1]

II. Observations on the Proportion of Viable Organisms, the Cytology, the Virulence, and the Antigenicity at Different Stages of Growth of BCG

FRANK FENNER AND RONALD H. LEACH

(Received for publication April 17, 1953)

INTRODUCTION

The previous paper (28) recorded the growth curves of virulent human-type tubercle bacilli (strain H37Rv) in Tween-albumin liquid medium and included observations on the cytology and virulence of the bacilli at different stages of growth.

BCG, like other mammalian tubercle bacilli, grows well in the Tween-albumin medium (29). Strains cultured and grown in this way retained the same degree of attenuation as they possessed before such passage (30). Furthermore, cultures grown in such media were shown to retain their viability and antigenicity when stored as liquid cultures in the refrigerator (31) and at room temperature (32), and they underwent freeze-drying with a relatively slight change in the proportion of viable cells (32).

It was pointed out elsewhere (33) that before growth of BCG in the Tween-albumin medium could be accepted as a satisfactory method of preparing the vaccine, a number of points required investigation. These included a study of the effect of the age of the culture on the virulence and antigenicity of the bacilli, in view of Bloch's demonstration (3) of the effect of age on the virulence of H37Rv.

The present work was undertaken to provide this information and also to broaden the basis of studies on the mode of growth of tubercle bacilli in liquid media.

MATERIALS AND METHODS

Most of the materials and methods employed have been described (28). In addition the following materials and methods were used:

Bacterial strain (BCG): A culture originated from a single colony of BCG with typical morphology (34) on oleic-acid-albumin-agar was used. It was kindly supplied by Dr. E. A. North and was derived from the substrain of BCG used for vaccine production in Australia, which in turn originated from culture F46 of the State Serum Institute, Copenhagen.

Culture media: The liquid medium was Tween-albumin medium, containing 0.05 per cent of Tween 80 and 0.5 per cent of bovine albumin fraction V. In most experiments glucose was omitted from the medium, but in some, which have been indicated, glucose was added to a final concentration of 0.5 per cent.

Guinea pigs: Stock bred in the Walter and Eliza Hall Institute was used, the animals being multicolored and weighing approximately 500 gm.

[1] From the Department of Microbiology, John Curtin School of Medical Research, The Australian National University, Canberra, Australia.

Nitrogen determinations: The bacterial cells were deposited from the culture by centrifugation, washed two or three times in distilled water (doubly distilled in glass apparatus) or with an aqueous 0.02 per cent solution of Tween 80. The deposit was then suspended in 0.75 ml. of 0.02 per cent Tween 80 solution and transferred to the digestion flask. A mixture of 1 gm. of $CuSO_4 \cdot 5H_2O$ and 1 gm. of SeO_2 per 100 ml. of concentrated sulfuric acid was used for digestion. A Markham Kjeldahl distillation apparatus was used, the boric acid titration method (35) being followed. Estimations were performed in triplicate, and appropriate blank titrations were made with diluent and uninoculated culture medium.

Virulence and antigenicity for mice: Twenty-five mice were inoculated intravenously with 0.1 ml. of a suspension containing approximately 9×10^7 bacterial units per ml. Approximately five weeks later 4 mice of each batch of 25 were sacrificed and the size and number of foci on the posterior surface of the lungs and the size of the spleen were determined. The left lungs were removed and sectioned to provide an objective measure of the extent of consolidation in the lungs. On the following day, 20 of the remaining 21 mice were inoculated, along with 20 normal controls, with 0.1 ml. of a suspension of a 4-day-old culture of H37Rv which contained 60 to 70×10^6 bacterial units per ml., counted in the Petroff-Hausser chamber and checked by viable counts. The mice were inoculated in six batches, the inoculum being of comparable density and the same age in each instance.

Virulence for guinea pigs: Following Jensen (36) and Suter and Dubos (37), an attempt was made to compare the virulence of cultures of varying age by the inoculation intracutaneously into guinea pigs of a series of tenfold dilutions of the cultures, each standardized to contain the same number of bacterial units in the initial tube. The size of the nodule produced and its progress from inoculation to healing were followed by weekly examinations.

EXPERIMENTAL PROCEDURE

Preliminary experiments indicated that, if accurate total counts and turbidity measurements were to be made during the early days of the culture period, it would be necessary to use heavy inocula. Two series of flasks were therefore inoculated with different dilutions of a 21-day-old culture of BCG grown in Tween-albumin medium which did not contain glucose. The culture was spun for three minutes at 1,000 r.p.m. in the angle centrifuge to deposit large clumps and was then found to contain no clumps of more than five bacilli each. The original culture (after spinning) contained about 2×10^8 bacterial units per ml. Thirty flasks, each containing 29 ml. of medium, were inoculated with 1.0 ml. of this suspension (*Experiment I*); and another thirty (*Experiment II*), with 1.0 ml. of a tenfold dilution of this in the filtrate through filter paper of another batch of the same original culture. The concentration of bacilli in the newly inoculated flasks is shown in table 1. Each flask was brought to 37°C. before inoculation, capped with Cellophane after inoculation, placed in an air incubator operating at 37°C., and agitated gently once daily. At intervals distilled water was added to each flask to maintain the volume at 30 ml.

At various intervals one flask was removed from one or both series and various tests performed. Total and viable counts and turbidity measurements were made immediately, and mice and guinea pigs were inoculated on the day that the flasks were removed. Films made then were stained in batches and the cell dispersion and staining properties determined. Material for microscopic examination was washed in alcohol, deposited in the centrifuge, resuspended in petrol-

ether, and stored in the refrigerator until opportunity offered to make and examine the films stained by Robinow's method. Aliquots of the cultures were stored in the refrigerator and all nitrogen determinations were carried out simultaneously, as were the neutral red tests.

The viable counts obtained during these two experiments were rather inconsistent, and frequently some of the drops deposited on the culture medium produced typically large colonies while others on the same plate failed to grow or produced minute colonies only. In order to check the ratio of viable bacilli to the total count, another experiment similar to *Experiment II*, but with a larger inoculum, was carried out (*Experiment III*). After centrifugation to remove larger clumps, 1 ml. of a 21-day-old culture of BCG, grown in Tween-albumin medium without glucose, was inoculated into 29 ml. of the same medium in a 250 ml. Erlenmeyer flask and incubated at 37°C. The concentration of the inoculum was adjusted so that the flasks contained initially approximately 7×10^6 bacterial units per

TABLE 1

THE TOTAL COUNTS (BACTERIAL UNITS PER ML.) AND ABSORPTIOMETER READINGS OF THE FRESHLY INOCULATED FLASKS

EXPERIMENT NUMBER	AGE OF INOCULUM	RATIO: VOLUME OF INOCULUM / VOLUME OF MEDIUM IN FLASKS	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^6)	ABSORPTIOMETER READING (× 1,000)
	days			
I	21	1/29	93	14
II	21	1/29	12	0 until third day
III	21	1/29	70	12
IV A	3	5/25	70	36
IV B	3	5/25	80	42
V A	21	5/25	176	30
V B	21	5/25	185	27

ml. At daily intervals for the first week, and then at nine, eleven, fourteen, twenty-one, and twenty-eight days, determinations were made of the total and viable counts, the opacity, and the degree of clumping.

To enable more accurate estimations to be made of the growth constants, with inocula of different ages and with slightly different media, another series of experiments was carried out with large inocula of bacilli which had been grown for either three or twenty-one days in liquid medium which contained no glucose. In one series of experiments the culture medium which was inoculated contained 0.5 per cent of glucose (IV A and V A), and in the other (IV B and V B) there was none. In each of these experiments, 250 ml. Erlenmeyer flasks containing 25 ml. of the appropriate medium were inoculated with 5 ml. of culture. The inoculum had been freed of large clumps by preliminary centrifugation and was so diluted or concentrated that it contained approximately 10^8 bacterial units per ml. Samples of approximately 8 ml. were taken from the flasks four, eight, twelve, sixteen, twenty, twenty-four, forty, forty-eight, and seventy-two hours after inoculation, and after seven, fourteen, and twenty-one days. The initial

counts of the inoculated flasks are shown in table 1. The optical density, total count, and state of dispersion of the bacilli were determined with each sample.

RESULTS

The results are condensed in tables 2 to 6 so that different investigations can be more easily compared.

Bacterial Growth Phases

The data which bear upon the bacterial growth phases were converted to $\log_2$ values and are shown graphically in figures 1, 2, 3, 4, and 5. In these experiments the state of aggregation of the bacilli (clumping factor) varied to a greater extent than in the experiments with H37Rv. It was found that the curve for total bacilli, obtained by multiplying the total counts (bacterial units) by the clumping factor, was more regular than the curve for total counts, and it also was therefore used in the determination of certain of the growth constants.

Straight lines were fitted by the method of least squares to all data in the interval during which the rate of increase appeared to be exponential from inspection of the graphs. The following growth constants (10) were determined from the regression equations (R and G) on the graphs T_1 and L).

$$\text{Exponential growth rate } (R) = \frac{\log_2 x_2 - \log_2 x_1}{t_2 - t_1}$$

$$\text{Generation time } (G) = \frac{1}{R} \text{ hours}$$

$$\text{Lag time } (T_1) = t_r - \frac{\log_2 x_r - \log_2 x_o}{R}$$

$$\text{Growth lag } (L) = T_1 . R.$$

x_o, x_r, x_1, x_2 are the optical densities (or counts) at times (in hours) o, r, t_1, and t_2.

Exponential growth rate and generation time: The exponential growth rate and its reciprocal, the generation time, varied considerably in the different experiments. If the generation times determined when the bacterial density was measured are first considered, i.e., the optical density of the culture, the values varied from fourteen hours (*Experiment II*) to thirty-six hours (*Experiment V A*). These two extreme values, however, occurred in experiments in which the data were not really adequate for an accurate determination of the period of exponential growth. In the majority of experiments, the generation time was approximately twenty hours. It is apparent from inspection of figures 1 and 3 that the duration of exponential growth differed considerably in *Experiments I* and *III*, which were carried out at different times but with identical inocula. The yield at fourteen days was approximately the same in the two experiments, but a high yield was attained much more rapidly in *Experiment I* than in *Experiment III*. It is difficult to suggest an explanation of these discrepancies.

TABLE 2

THE RESULTS OF EXPERIMENT I. FLASKS CONTAINING 29 ML. OF MEDIUM WERE INOCULATED WITH 1 ML. OF TWENTY-ONE-DAY-OLD CULTURE CONTAINING 2 × 10^8 BACTERIAL UNITS PER ML.

AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^3)		VIABLE COUNT (BACTERIAL UNITS PER ML. × 10^3)		RATIO: VIABLE COUNT / TOTAL COUNT × 100	CLUMPING FACTOR	TOTAL BACILLI PER ML. × 10^3 (TOTAL COUNT × CLUMPING FACTOR)	OPTICAL DENSITY (SCALE READING × 1,000)	NITROGEN CONTENT (γ PER ML.)	AVERAGE LENGTH OF BACILLI	AVERAGE NUMBER OF NUCLEI PER BACILLUS	NONACID-FAST BACILLI	VIRULENCE		IMMUNOGENICITY (MOUSE)
	Mean	Standard Error	Mean	Standard Error									Guinea Pig	Mouse	
										(μ)		*per cent*			
0 hours	93	4.04	48	2.00	51	2.8	261	14	2.2	2.5	3.4	6.0*	11†	15, +‡	3.2§
4½	81	3.98	52	2.48	64	2.0	164	17	1.6	—		1.0			
8	79	4.41	44	0.93	55			17	1.5	2.5	3.0	—			
12	85	3.85	33	1.99	38			23	1.2	—	—	—			
16	81	—	—					24	2.0	—	—	—			
20	109	—	—					27	1.6	—	—	—			
22	91	—	—			4.3	391	30	—	3.8	3.8	2.5			
24	71	3.98	31	2.33	43	4.9	348	33	—	—	—	8.0	13	15, +	3.3
26	101	8.23						36	—	—	—	—			
28	107	5.54						30	—	—	—	—			
30	140	8.44						37	—	—	—	—			
37½	96	5.02						56	2.3	—	—	—			
48	135	6.42	83	6.22	61	6.5	883	79	—	3.4	3.4	1.0	13	10, +	2.5
61½								119	4.5	—	—	—			
3 days	780	20.59	640	38.16	82	4.5	3,530	228	6.4	3.1	3.3	0.5	12	15, +	3.4
4	680	19.70	740	37.53	108	6.6	4,490	383	9.9	2.6	3.3	0.0	11	20, ++	3.0
5	790	16.99	610	25.69	77	9.6	7,580	450	16.5	2.6	2.5	0.0	10	40, ++	3.0
6	1,500	43.60	990	57.60	66	5.0	7,500	530	20.6	2.5	2.5	0.0	12	40, +	2.3
7	1,020	49.43	630	58.80	62	7.8	7,960	660	22.2	2.3	2.6	0.0	12	50, +	3.5
8	1,590	45.34	750	32.47	47	5.4	8,510	770	23.2	2.0	1.8	0.0	12	50, +	2.8
11	6,500	100.3	2,250	131.4	35	3.0	19,180	950	30.9	2.2	2.4	0.0	11	15, ±	3.1
14	9,050	228.6	1,260	90.0	14	2.7	24,000	970	29.7	1.8	2.0	0.0	10	15, ±	2.8
21	6,020	202.2	—	—		4.1	24,900	1,080	30.8	1.8	2.0	0.0	7	15, +	2.6
28	4,580	206.2	—	—		3.8	17,200	1,090	28.1	1.7	2.0	0.0	8	10, −	2.4

* Figures indicate the percentage of bacterial units in which there was at least one nonacid-fast tubercle bacillus.

† 11 et cetera = diameter in mm. of nodule produced by the inoculation of approximately 5 × 10^5 bacterial units, measured two weeks after inoculation.

‡ 15, + et cetera: 15 = approximate number of foci on posterior surface on left lung, ± to ++++ = arbitrary scale of splenic enlargement.

§ 3.2 et cetera = $\frac{\text{M.D.T. 50\% vaccinated}}{\text{M.D.T. 50\% control}}$

‖ Unsatisfactory batch of culture medium used for viable counts.

TABLE 3

THE RESULTS OF EXPERIMENT II. FLASKS CONTAINING 29 ML. OF MEDIUM WERE INOCULATED WITH 1 ML. OF THE SAME SUSPENSION AS THAT USED FOR EXPERIMENT I DILUTED SO THAT IT CONTAINED APPROXIMATELY 4×10^7 BACTERIAL UNITS PER ML.

AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. $\times 10^6$)		VIABLE COUNT (BACTERIAL UNITS PER ML. $\times 10^6$)		RATIO: $\frac{\text{VIABLE COUNT}}{\text{TOTAL COUNT}} \times 100$	CLUMPING FACTOR	TOTAL BACILLI PER ML. $\times 10^6$ (TOTAL COUNT $\times$ CLUMPING FACTOR)	OPTICAL DENSITY (SCALE READING $\times 100$)	NITROGEN CONTENT (γ PER ML.)	AVERAGE LENGTH OF BACILLI	AVERAGE NUMBER OF NUCLEI PER BACILLUS	NONACID-FAST BACILLI	VIRULENCE		IMMUNOGENICITY (MOUSE)
	Mean	Standard Error	Mean	Standard Error									Guinea Pig	Mouse	
days										μ		*per cent*			
0	12.5	1.29	4.7	2.46	38	3.0	37.9	—	—	2.2	2.5	4	—	—	—
1	10.3	1.15	3.3	0.36	32	3.9	39.8	—	—	3.4	4.2	10	—	—	—
2	15.0	1.21	9.1	0.40	61	5.2	78.0	—	—	3.6	4.2	30	—	—	—
3	26.0	1.63	15.8	0.68	60	7.0	182.0	11	—	3.3	3.7	41	—	—	—
4	36.0	2.27	27.0	1.66	75	8.3	300.0	40	3.4	3.5	4.4	11	—	—	—
5	134.0	8.74	96.0	5.61	72	7.0	954.0	125	6.1	3.2	3.9	2	—	—	—
6	382.0	15.85	212.0	10.57	55	8.2	3,130.0	245	7.1	2.3	2.6	0	13	50, +	1.9
7	1,510.0	36.98	780.0	39.21	52	3.7	5,520.0	465	15.0	2.3	2.1	0	10	50, +	3.3
8	838.0	46.89	645.0	34.46	76	9.9	8,250.0	640	21.9	2.1	2.1	0.5	12	40, +	3.0
9	1,020.0	45.98	619.0	60.82	61	13.5	13,720.0	700	22.0	2.0	2.0	0	12	50, +	2.5
10	1,390.0	46.42	1,080.0	59.49	77	9.0	12,500.0	840	—	1.8	1.9	0.5	11	50, +	3.0
11	3,000.0	70.7	2,610.0	76.86	87	6.2	18,460.0	830	27.2	1.9	1.7	0	13	20, ±	2.1
12	5,100.0	140.7	2,200.0	135.3	43	5.0	25,400.0	1,100	30.0	1.6	1.5	0	12	—	—
13	8,330.0	168.9	8,800.0	626.3	106	2.9	24,330	1,070	28.3	1.8	1.9	3	6	20, ±	2.4
14	8,050.0	309.0	2,370.0	153.64	29	3.4	27,230	1,040	27.0	1.8	2.0	0	10	5, ±	2.8
21	10,020.0	246.6	4,270.0	472.0	43	2.6	26,040	990	31.5	1.8	2.1	0	7	2, —	2.4
28	8,750	199.5	4,970.0	190.4	56	2.2	19,520	900	34.1	1.8	2.1	0	8	5, —	2.6

Symbols as in table 2.

Owing to the dilute nature of the inoculum in *Experiment II*, absorptiometer readings were not possible until the third day. If the values for the third, fourth, and fifth days were used, the generation time was quite short (fourteen hours), but it is possible that the lack of readings earlier than the third day made this an underestimation.

Detailed data were available for *Experiments IV* and *V* for the first three days only. In all of these experiments it is possible that somewhat different values for the growth constants would have been obtained if figures had been available for the fourth and fifth days. This applies particularly in *Experiment V A*. The freshly inoculated flasks in *Experiment IV* contained approximately the same number of bacterial units as in *Experiments I* and *III*, but the optical density

TABLE 4

THE RESULTS OF EXPERIMENT III. FLASKS CONTAINING 29 ML. OF MEDIUM WERE INOCULATED WITH 1 ML. OF A TWENTY-ONE-DAY-OLD CULTURE CONTAINING APPROXIMATELY 2 × 10^8 BACTERIAL UNITS PER ML.

AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^3)		VIABLE COUNT (BACTERIAL UNITS PER ML. × 10^3)		RATIO: $\frac{\text{VIABLE COUNT}}{\text{TOTAL COUNT}} \times 100$	CLUMPING FACTOR	TOTAL BACILLI PER ML. × 10^3 (TOTAL COUNT × CLUMPING FACTOR)	NON-ACID-FAST BACILLI	OPTICAL DENSITY (SCALE READING × 1,000)
	Mean	Standard Error	Mean	Standard Error					
days								*per cent*	
0	73	5.302	49	2.595	67	1.7	124	5	12
1	92	8.065	34	1.569	37	2.2	202	11	26
2	77	7.04	93	5.297	121	3.1	239	3	32
3	119	11.64	62	6.835	52	2.6	309	7	67
4	167	6.73	138	5.855	83	5.2	868	0	156
5	389	14.6	313	19.41	81	4.3	1,670	0	253
6	198	12.76	318	13.54	161	8.5	1,680	0	355
7	831	12.74	656	48.86	79	4.6	3,820	0	480
9	1,040	34.36	—		—	7.3	7,590	0	610
11	970	44.98	981	43.50	101	6.2	6,010	0	796
14	1,530	93.9	—		—	4.4	6,730	0	950
21	6,690	330.4	9,630	309.4	144	2.9	19,400	0	1,180
28	8,050	275.5	4,790	316.7	55	2.2	17,700	0	—

was considerably higher, due to the large size of the young bacterial cells. The generation times in both portions of *Experiment IV* (with and without glucose in the medium) were a little more than twenty hours. With the 21-day-old inoculum the generation time was somewhat longer, especially in *Experiment V A* (21-day-old inoculum, medium lacking glucose), in which it was thirty-six hours; but it is doubtful if the data available in this experiment were adequate for the determination of the generation time.

In the first five experiments it was possible to estimate the generation time by consideration of the numbers of bacterial units and the total number of bacilli. The results obtained agreed reasonably well with determinations based upon the opacity, considering the errors involved in both the total counts and the estimation of a clumping factor. In *Experiment IV* the generation time estimated in

terms of total bacilli was not very much different from the generation time determined from the absorptiometer measurements. In *Experiment V* it is apparent from inspection of the graphs that there was no increase in the total counts and only a very slight increase in the figures for total bacilli during the first three days. Estimation of the generation time was not justified in these instances.

TABLE 5

THE RESULTS OF EXPERIMENT IV. FLASKS CONTAINING 25 ML. OF MEDIUM WERE INOCULATED WITH 5 ML. OF A THREE-DAY-OLD CULTURE CONTAINING APPROXIMATELY 4 × 10^7 BACTERIAL UNITS PER ML.

EXPERIMENT	AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^6)		VIABLE COUNT (BACTERIAL UNITS PER ML. × 10^6)		CLUMPING FACTOR	TOTAL BACILLI PER ML. × 10^6 (TOTAL COUNT × CLUMPING FACTOR)	NON-ACID-FAST BACILLI	OPTICAL DENSITY (SCALE READING × 1,000)
		Mean	Standard Error	Mean	Standard Error				
								per cent	
	0 hours	73	5.59	79	10.88	2.8	203	5	36
	4	115	8.22			2.3	264	2	44
	8	139	6.88			2.7	375	8	49
	12	132	8.04			2.2	290	31	60
IV A	16	110	10.7						65
medium does	20	154	12.71			3.5	539	28	74
not contain	24	214	9.16			2.1	428	5	94
glucose	40								154
	48	460	25.26			2.3	1,060	0	177
	3 days	888	22.12			2.3	2,040	2	313
	7	1,060	37.58			2.3	2,438	0	980
	14	9,050	478.2	132	162.0	2.2	19,900	0	2,430
	21	14,400	866.9	6,820	296.0	1.6	23,000	0	2,560
	0 hours	75	3.83	48	1.033	2.8	210	25	42
	4	139	7.3			2.7	372	15	50
	8	176	10.57			2.1	370	9	53
	12	148	8.34			2.3	339	2	65
IV B	16	94	7.95						66
medium con-	20	200	16.81			2.8	560	13	88
tains glucose	24	372	14.78			2.5	933	12	94
	40	—							162
	48	275	16.24			3.7	1,020	8	178
	3 days	550	13.45			4.0	2,200	0	324
	7	1,250	35.8			4.9	6,110	0	1,200
	14	6,900	203.1	5,780	280.0	3.8	26,220	0	2,630
	21	8,560	158.6	8,700	515.0	3.4	29,100	0	3,200

The lag phase: The duration of the lag phase varied considerably, depending upon whether it was determined from estimations of the bacterial density or counts of the bacilli. There is little point in discussing the figures obtained from consideration of counts of the bacterial units, for these were complicated by the degree of clumping at different stages of growth.

In *Experiments I* and *III* the lag phase, in terms of optical density, was much

shorter than when total bacilli were considered. This result is in agreement with numerous observations upon other bacteria, namely, that increase in size precedes increase in numbers by an appreciable interval.

In *Experiment IV*, in which a 3-day-old inoculum was used, there was no lag either in growth of the bacilli or in their multiplication. This accords with the observations on other bacteria transferred to new media during the exponential

TABLE 6

THE RESULTS OF EXPERIMENT V. FLASKS CONTAINING 25 ML. OF MEDIUM WERE INOCULATED WITH 5 ML. OF A TWENTY-ONE-DAY-OLD CULTURE CONTAINING APPROXIMATELY 10^9 INFECTIVE PARTICLES PER ML.

EXPERIMENT	AGE OF CULTURE	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^9)		VIABLE COUNT (BACTERIAL UNITS PER ML. × 10^9)		CLUMPING FACTOR	TOTAL BACILLI PER ML. × 10^9 (TOTAL COUNT × CLUMPING FACTOR)	NON-ACID-FAST BACILLI	OPTICAL DENSITY (SCALE READING × 1,000)
		Mean	Standard Error	Mean	Standard Error				
								per cent	
	0 hours	176	8.13	76	7.52	2.5	440	0	30
	4	192	6.72			2.9	556	0	34
	8	198	9.46			2.3	455	0	35
V A	12	190	4.48			2.1	398	0	37
does not contain glucose	16	203	10.15			1.9	383	0	41
	20	197	9.64			2.1	413	0	45
	24	209	9.98	200	9.0	2.1	439	0	47
	48	202	9.05	109	4.91	2.8	552	0	74
	3 days	216	13.2	165	11.86	4.5	972	5	133
	7	475	22.9	505	74.3	7.2	3,420	0	525
	14	9,500	593.0	—		2.2	20,890	0	1,530
	0 hours	185	12.62	107	14.11	2.5	462	0	27
	4	164	8.91			1.8	295	2	32
	8	193	10.47			1.6	309	0	34
	12	157	9.84			2.3	361	0	34
V B	16	185	9.85			1.9	350	0	37
contains	20	188	7.18			2.0	376	3	35
glucose	24	189	7.98			2.0	378	3	45
	48	211	9.71	114	5.98	3.1	654	0	93
	3 days	194	11.99	207	16.67	4.9	852	3	167
	7	2,270	79.2	—		2.8	6,360	2	940
	14	13,125	701.6	—		2.3	30,200	0	2,980

phase of growth (38). In *Experiment V* (21-day-old inoculum), on the other hand, there was no appreciable change in the number of bacterial units, and only a slight change in the total numbers of bacilli during the first three days, although there was an appreciable increase in the bacterial density.

The phase of retardation: The occurrence of arithmetic linear growth of tubercle bacilli was first pointed out by Fisher, Kirchheimer, and Hess (13). Kirchheimer and Fisher (14) have stated that it occurs among all types of *Mycobacteria* grown in liquid media with Tween 80. Arithmetic plotting of growth in *Experiments I*,

II, and *III* indicated that, as with H37Rv, there was a phase of arithmetic linear growth corresponding approximately to the retardation phase. As pointed

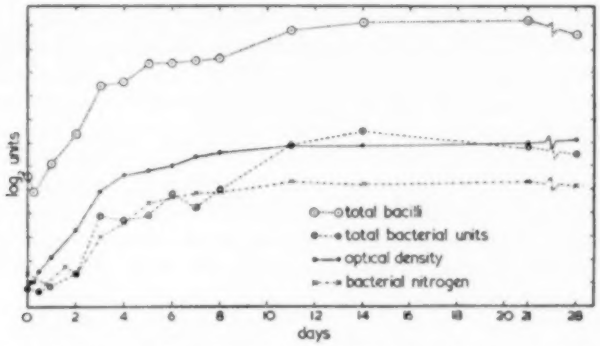

FIG. 1. Growth curves in *Experiment I*. Ordinates: $\log_2$ values for optical density, the total count of bacterial units per ml., the total bacilli per ml., and the bacterial nitrogen concentration of cultures incubated for periods varying from twelve hours to twenty-eight days. Values for optical density adjusted so that the initial value coincides with that of the total count (bacterial units). Total bacilli per ml. equal total bacterial units per ml. (counted in Petroff-Hausser chamber) × clumping factor (average number of bacilli per bacterial unit).

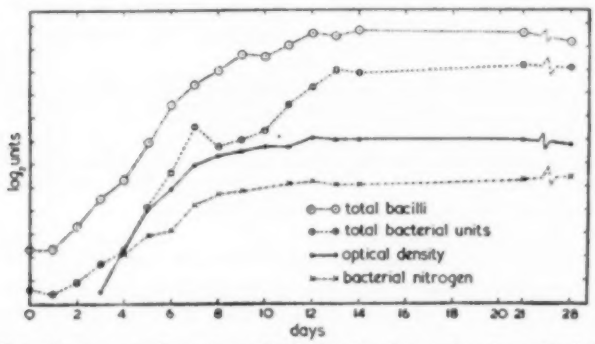

FIG. 2. Growth curves in *Experiment II*. Ordinates: $\log_2$ values for optical density, total count of bacterial units per ml., the total bacilli per ml., and the bacterial nitrogen concentration of cultures incubated for periods varying from twelve hours to twenty-eight days. With the small inoculum, it was not possible to determine the optical density or the bacterial nitrogen concentration until the third day of the experiment. Total bacilli per ml. equal total bacterial units per ml. (counted in Petroff-Hausser chamber) × clumping factor (average number of bacilli per bacterial unit).

out above, exponential growth ceased in *Experiment I* at the end of the third day, and from the third to the eighth days growth (determined by opacity measurements) passed through an arithmetic linear phase before becoming stationary. In *Experiment II*, exponential growth continued until the end of

the fifth day, and the stage of arithmetic linear growth extended from the sixth to the eleventh days.

The curves were not as smooth as those obtained with H37Rv, but there is little doubt that they reflect the same phenomenon. In figure 6 the optical density, the total bacilli, and the nitrogen content in *Experiment II* are all plotted arithmetically. From about the end of the fifth day, when exponential growth ceased, arithmetic linear growth commenced and extended from the sixth to the twelfth days.

Yield after incubation for various periods: The yield of bacteria after various periods of incubation can be expressed either in terms of the bacterial mass (represented by the optical density) or the number of bacteria (total count [bacterial units] or, better, total bacilli). The yields at seven, fourteen, and twenty-one days after inoculation in all of the experiments with BCG are shown in table 8.

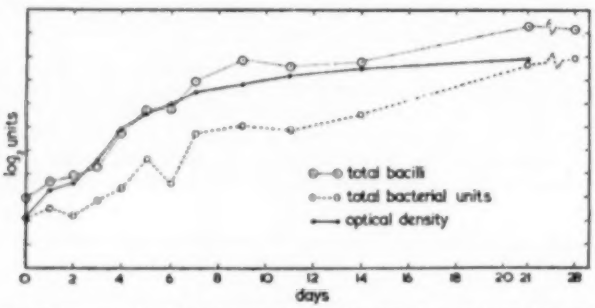

FIG. 3. Growth curves in *Experiment III*. Ordinates: $\log_2$ values for optical density, total count of bacterial units per ml., and the total bacilli per ml. of cultures incubated for periods varying from one to twenty-eight days. Values for optical density adjusted so that the initial value coincides with that of the total count (bacterial units). Total bacilli per ml. equal total bacterial units per ml. (counted in Petroff-Hausser chamber) × clumping factor (average number of bacilli per bacterial unit).

As can be judged from the growth curves (figures 1, 2, and 3), the yield approaches its maximum value by the end of the seventh day. The bacterial mass was doubled during the next seven days in some instances but showed smaller increases in others. The increase in the number of bacteria was greater, a reflection of the decrease in their average size. The discrepancy between total bacilli and total bacterial units diminished, due to the greater dispersion of the older cultures. There was very little change between the fourteenth and twenty-first days.

The yield at fourteen days was much the same in *Experiments I*, *II*, and *III*, although the inoculum was considerably smaller in *Experiment II*. The yield in *Experiments IV* and *V* (in terms of optical density) was considerably greater than in *Experiments I*, *II*, and *III*, but the numbers of bacilli were approximately the same in all of the experiments.

Dubos and Fenner (29) found that the addition of glucose to the medium

enhanced the growth of BCG, and in *Experiments IV* and *V* (tables 5 and 6) this effect is apparent. The yield at seven days, whether measured by the total bacterial units, the total bacilli, or the optical density, was considerably higher in the cultures containing glucose, and the disproportion was still evident in cultures fourteen and twenty-one days old.

TABLE 7

GROWTH CONSTANTS OF BCG GROWN IN TWEEN-ALBUMIN MEDIUM

EXPERIMENT	PROPERTY MEASURED	INTERVALS	EXPONENTIAL GROWTH RATE (R)	GENERATION TIME ($1/R$)	LAG TIME (T_1)	GROWTH LAG (L)
		hours		*hours*	*hours*	
I	Opacity	30–72	0.060	16.7	6	0.36
	Total bacilli	24–72	0.069	14.5	18	1.3
	Total bacterial units	26–72	0.064	15.6	28	1.7
	Bacterial nitrogen	48–120	0.042	24	44	1.9
II	Opacity	72–120*	0.073	14	24	1.74
	Total bacilli	24–144	0.051	20	27	1.43
	Total bacterial units	48–168	0.056	18	50	2.44
III	Opacity	48–120	0.043	23	13	0.58
	Total bacilli	48–120	0.041	24	32	1.30
	Total bacterial units	48–120	0.031	32	47	1.48
IV A (3-day-old inoculum; no glucose)	Opacity	8–40	0.049	20	0	0.0
	Total bacilli	8–48	0.043	23	0	0.0
	Total bacterial units	8–48	0.046	22	0	0.0
IV B (3-day-old inoculum; with glucose)	Opacity	8–48	0.044	23	0	0.0
	Total bacilli	8–72	0.040	25	0	0.0
V A (21-day-old inoculum; no glucose)	Opacity	8–48*	0.028	36	0	0.0
V B (21-day-old inoculum; with glucose)	Opacity	12–48	0.041	24	8	0.24

* Data not adequate for an accurate estimate of the period during which exponential growth occurred.

Correlation with the nitrogen content: There was a reasonably close parallel between the optical density and the total nitrogen content in *Experiment I* after the first two days. During the first two days, however, the nitrogen content remained virtually unchanged while the optical density increased exponentially. The small inoculum used in *Experiment II* makes comparison during this early period impossible.

The relationship of the viable and total counts: Viable counts were made throughout *Experiments I*, *II*, and *III*. There were many inconsistencies in replicate

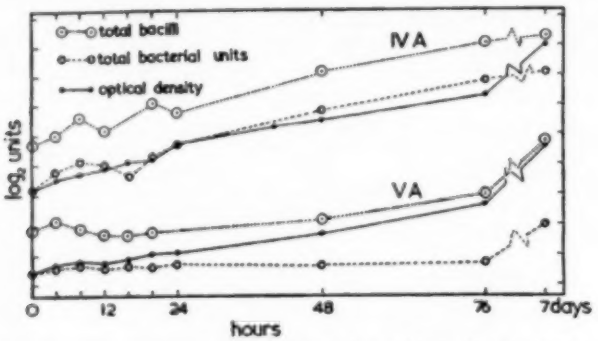

FIG. 4. Growth curves in *Experiments IV A* (3-day-old inoculum) and *V A* (21-day-old inoculum). No glucose in culture medium. Ordinates: $\log_2$ values of optical density, total count of bacterial units per ml., and the total bacilli per ml. of cultures incubated from two hours to seven days. Values for optical density adjusted so that initial values coincide with those of total count (bacterial units). Total bacilli per ml. equal total bacterial units per ml. (counted in Petroff-Hausser chamber) × clumping factor (average number of bacilli per bacterial unit).

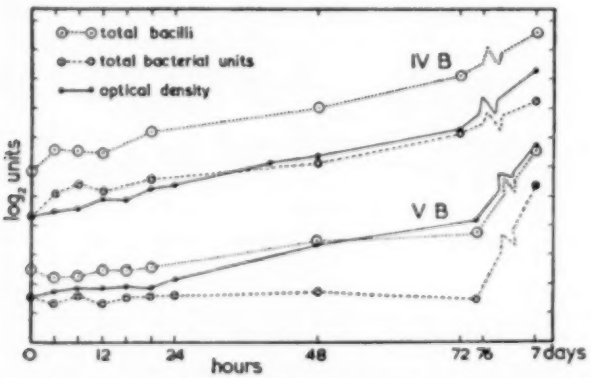

FIG. 5. Growth curves in *Experiments IV B* (3-day-old inoculum) and *V B* (21-day-old inoculum); 0.5 per cent glucose in medium. Ordinates: $\log_2$ values of optical density, total count of bacterial units per ml., and the total bacilli per ml. of cultures incubated from two hours to seven days. Values for optical density adjusted so that initial values coincide with those of total count (bacterial units). Total bacilli per ml. equal total bacterial units per ml. (counted in Petroff-Hausser chamber) × clumping factor (average number of bacilli per bacterial unit).

inoculated plates in the first two experiments. These did not occur in *Experiment III*. In all cases, however, the ratio of the viable and total counts varied between 50 and 100 per cent. Considering the inaccuracies of this ratio (2), it appears that with BCG, as with H37Rv, the majority of the bacilli in the culture are

viable (as evidenced by their ability to grow on the culture medium) at all stages of growth.

It was reported earlier (29) that the proportion of viable bacilli dropped considerably in the later stages of incubation (twenty-one and twenty-eight days) of cultures of BCG containing glucose. In *Experiments IV* and *V* the attempt to confirm this was nullified by contamination of the culture flasks in the last week. Up to the twenty-first day in *Experiment IV* (3-day-old inoculum), there

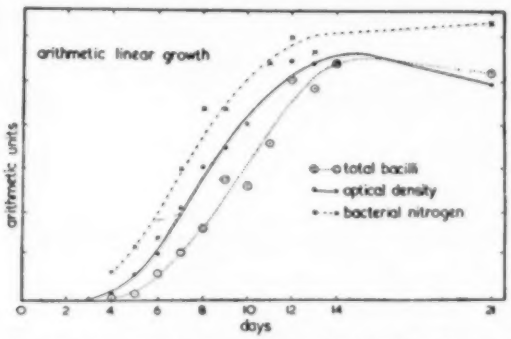

FIG. 6. Growth curves in *Experiment II*, plotted arithmetically. Ordinates: values on arithmetic scale for total bacilli per ml., optical density, and bacterial nitrogen. Total bacilli per ml. equal total bacterial units per ml. (counted in Petroff-Hausser chamber) × clumping factor (average number of bacilli per bacterial unit).

TABLE 8

THE YIELD AT VARIOUS INTERVALS AFTER INOCULATION, EXPRESSED AS TOTAL COUNT (BACTERIAL UNITS PER ML.), TOTAL BACILLI, AND OPTICAL DENSITY

EXPERIMENT	TOTAL COUNT (BACTERIAL UNITS PER ML. × 10^3)			TOTAL BACILLI PER ML. (× 10^3)			OPTICAL DENSITY (SCALE READING × 1,000)		
	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
I	10.2	90.5	60.2	79.6	240	249	660	970	1,080
II	15.1	80.5	100	55.2	272	260	465	1,040	990
III	9.7	15.3	66.9	60.2	67	194	796	950	1,180
IV A	10.6	90.5	144	24.4	199	230	980	2,430	2,560
IV B	12.5	69	85.6	61.1	262	291	1,200	2,630	3,200
V A	4.7	95	—	34.2	209	—	525	1,530	—
V B	22.7	131	—	63.6	302	—	940	2,980	—

was no drop in the proportion of viable organisms. With the 21-day-old inoculum (*Experiment V*), there was a fall in the proportion of viable bacilli at fourteen days in the culture medium containing glucose, but no change at twenty-one days in the culture lacking glucose.

Observations on the Bacterial Cytology

The sizes of the bacilli were measured, their state of aggregation was determined, nuclear counts were made in some of the experiments, and the occurrence of nonacid-fast bacilli was also noted.

Size of the bacilli: The average length measurements of bacilli at different stages of growth in *Experiments I* and *II* are shown in the upper portions of figures 7 and 8. The variation in the size of the bacilli was considerable and is better shown by the curves indicating the percentage of small bacilli (less than 2.3μ long) and long bacilli (more than 3.5μ long). In both instances there was, within a day of inoculation of the flask, an almost complete disappearance of small bacilli and

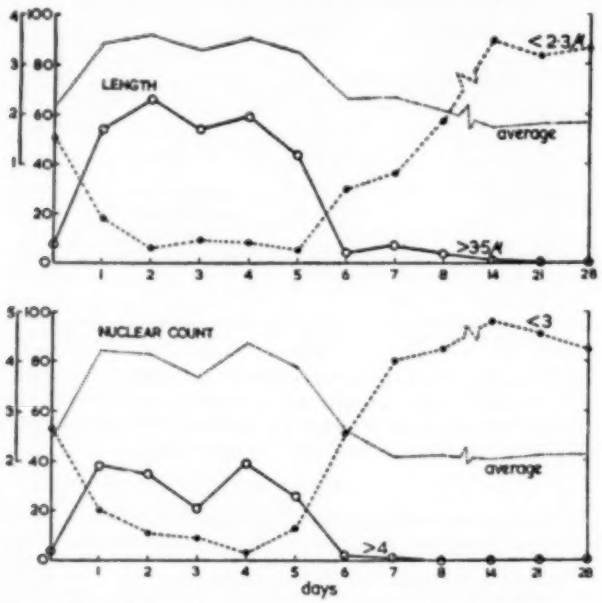

FIG. 7. (Upper) The changes in average length of the bacilli and the percentages of small (less than 2.3μ) and large (more than 3.5μ) bacilli at various intervals after inoculation of the culture flasks in *Experiment I*. Ordinates: 1, 2, 3, et cetera = average length of bacilli (μ); 20, 40, et cetera = percentage of short and long bacilli.

(Lower) The changes in the average numbers of nuclei per bacillus and the percentages of bacilli with few (less than 3) or many (more than 4) nuclei at various intervals after inoculation of the culture flasks in *Experiment I*. Ordinates: 1, 2, 3, et cetera = average number of nuclei per bacillus; 20, 40, et cetera = percentages of bacilli with few and many nuclei.

their replacement by rather long organisms. The peaks were sharper in *Experiment I*, in which the maximum values were reached at twenty-four hours, than in *Experiment II*, in which there was a sustained rather high level of the curves for the average length and long bacilli from the first to the fifth days, inclusive. This difference is related to the larger initial inoculum and the shorter period of exponential growth in *Experiment I*. Comparison of figures 1 and 7 and figures 2 and 8 shows that there was a close correlation between the growth phases and the size and nuclear development of the bacilli. At the end of the first week, the

proportion of large and small bacilli changed considerably; and from the fourteenth day onwards the values were stable, the great majority of the bacilli being short (less than 2.3μ long) and the average length being approximately 2μ. The maximum average length was about twice this value. These observations agree reasonably well with the electronmicroscopic investigations of the growth of BCG described by Grasset and Bonifas (39).

The nuclear apparatus: The average numbers of nuclei,[2] and the proportion of bacilli with few (one or two) and many (more than four) nuclei in *Experiments I*

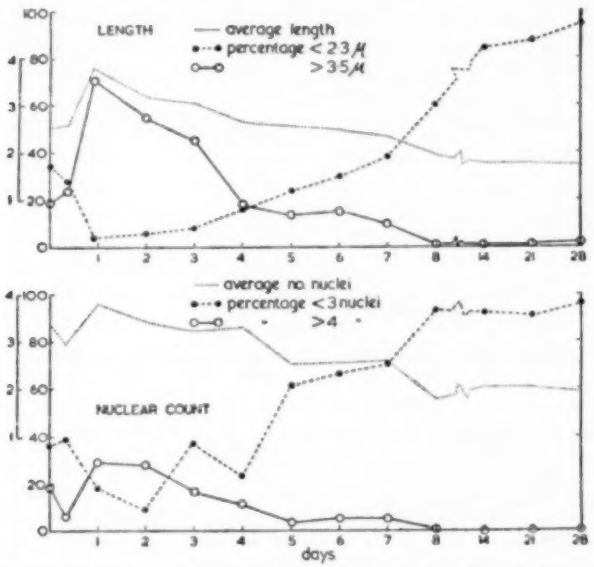

FIG. 8. (Upper) The changes in average length of the bacilli and the percentages of small (less than 2.3μ) and large (more than 3.5μ) bacilli at various intervals after inoculation of the culture flasks in *Experiment II*. Ordinates as in figure 7.

(Lower) The changes in the average numbers of nuclei per bacillus, and the percentages of bacilli with few (less than 3) or many (more than 4) nuclei at various intervals after inoculation of the culture flasks in *Experiment II*. Ordinates as in figure 7.

and *II* are shown graphically in the lower portions of figures 7 and 8. It is apparent that there is a close agreement between the measurements of the length of the bacilli and the results of the nuclear counts. The early stages of growth and the exponential phase are characterized by the development of many long multinucleate bacilli, whereas during the stationary phase the organisms are short, and there are few bacilli containing more than two nuclei. Uninucleate bacilli appear to be quite rare.

[2] The dark-staining areas seen when tubercle bacilli which have been subjected to the correct degree of acid hydrolysis are stained by Giemsa are regarded as bacterial nuclei (*see* text).

Aggregation of the bacilli: The state of aggregation of the bacilli was determined in each experiment, and the values of the average number of bacilli per bacterial unit, the clumping factor, are set out in the appropriate tables. The data on the state of aggregation in the first three experiments are shown in figure 9.

The position was much the same in the three experiments. From an initial condition in which there were virtually no large clumps, due to the preliminary

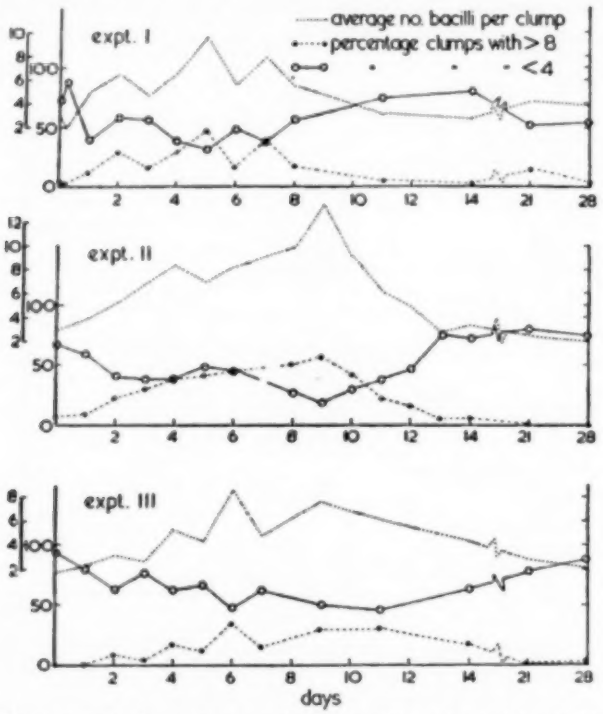

FIG. 9. The changes in the average numbers of bacilli per bacterial unit (the clumping factor) and the percentages of units containing few (one, two, or three) and those containing many (more than 8) bacilli at various intervals after inoculation of the culture flasks in *Experiments I, II,* and *III*. Ordinates: 2, 4, 6, et cetera = average number of bacilli per bacterial unit; 50, 100 = percentage of clumps containing few or many bacilli.

centrifugation, there was a gradual increase in the number of clumps containing larger numbers of bacilli and in the average number of bacilli per clump, so that these figures were relatively high (clumping factor between 8 and 10) between the fourth and the tenth days. By the fourteenth day the culture had become much more dispersed, and during the last fortnight large clumps virtually disappeared.

Degree of acid-fastness: Nonacid-fast bacilli were rarely encountered in these cultures and, when they did occur, they were usually found in young cultures

(less than five days old). The percentages of bacterial units in which there was at least one nonacid-fast bacillus are shown in the appropriate tables.

The virulence of cultures of varying age: The virulence of BCG is rather difficult to assess. Two methods were used by Suter and Dubos (37) to compare three substrains of BCG, namely, the persistence of the nodules produced by the intradermal inoculation of comparable doses into guinea pigs; and the lesions produced in the lungs of mice after the intravenous inoculation of standard doses. An attempt was made to assess the virulence of cultures of different ages by both of these methods.

Guinea pigs were inoculated intracutaneously with 0.1 ml. doses of graded tenfold dilutions of cultures of different ages, the inoculum being standardized by total counts so that the most concentrated (except in 21- and 28-day-old specimens) contained approximately 5×10^5 bacterial units. The inocula were obtained from the cultures of *Experiments I* and *II*. In table 9, the actual viable counts of the inocula, which do not differ greatly from 5×10^5, are shown. At weekly intervals the diameter of the nodules which developed at the various inoculation sites was measured. The persistence in weeks of these nodules is shown in table 9 as is also the diameter of those produced by the most concentrated inocula when they had attained their maximum size, two weeks after the inoculation. There were considerable variations in the persistence of the nodules and in the appearance of nodules in response to the inoculation of the dilution containing approximately 500 bacterial units. In no instance were nodules observed at the sites inoculated with either of the two higher dilutions used, which contained approximately 50 and 5 bacterial units per ml., respectively. The only systematic variation was the smaller size of the nodules produced by the 21-day-old and 28-day-old cultures. Although no satisfactory viable counts for these cultures were obtained in *Experiment I*, the results obtained in *Experiment II* indicate that this diminished reaction was not caused by a fall in the proportion of viable organisms.

Mice were inoculated intravenously with 0.2 ml. of cultures of various ages, so concentrated or diluted that the inoculum contained approximately 2×10^7 bacterial units. Five weeks later, 4 mice in each batch were killed and their lungs were examined macroscopically and microscopically. The results are not easy to express quantitatively, but are summarized in tables 2 and 3. During the first ten days of each experiment, practically identical results were recorded in each group of mice. There were between 20 and 50 foci of consolidation, approximately 1 to 2 mm. in diameter, on the posterior surface of each lung, and the spleen was slightly or moderately enlarged. With cultures more than ten days old the lesions became progressively less severe. Foci of consolidation in the lungs became less numerous and smaller, and the spleen was rarely enlarged. This change was particularly apparent in the cultures of *Experiment II* and was obviously not due to a decrease in the numbers of viable bacterial units, if viability was measured by the ability to grow on oleic-acid–albumin–agar medium.

The data from the experiments on both mice and guinea pigs are consistent, although the differences between cultures of different ages are small. Cultures

aged twenty-one or twenty-eight days appear to be less effective in producing persistent nodules in the skin of guinea pigs and in producing foci of pulmonary consolidation and enlargement of the spleen in mice. Young cultures in the exponential growth phase do not appear to have any enhanced virulence analogous

TABLE 9

THE PERSISTENCE OF THE NODULES PRODUCED BY THE INOCULATION OF VARIOUS DOSES OF BCG CULTURES OF VARIOUS AGES, AND THE AVERAGE DIAMETER AT TWO WEEKS OF THE NODULES PRODUCED BY THE MOST CONCENTRATED INOCULUM

EXPERIMENT	AGE OF CULTURE	VIABLE COUNT OF UNDILUTED INOCULUM (BACTERIAL UNITS INJECTED)	DILUTION OF INOCULUM				AVERAGE DIAMETER OF NODULES PRODUCED BY UNDILUTED INOCULUM, TWO WEEKS AFTER INOCULATION (IN MM.)
			Undiluted	10^{-1}	10^{-2}	10^{-3}	
	days						
I	0	5×10^5	6*	6	5	3	11u†
	1	3×10^5	6	5	4	2	13u
	2	4×10^5	6	7	6	5	13u
	3	7×10^5	6	6	3	2	12u
	4	8×10^5	5	5	7	2	11u
	5	6×10^5	5	5	7	0	10u
	6	7×10^5	6	6	7	0	12u
	7	4×10^5	7	5	4	0	12u
	8	5×10^5	6	6	8	0	12u
	11	4×10^5	8	8	8	0	11u
	14	1.4×10^5	7	7	6	5	10u
	21	—	7	8	8	0	7u (10u)‡
	28	—	6	6	0	0	8 (11u)
II	6	5×10^5	5	6	7	8	13u
	7	5×10^5	5	5	7	0	10u
	8	4×10^5	6	6	8	8	12u
	9	4×10^5	7	7	8	8	12u
	10	6×10^5	6	7	7	5	11u
	11	8×10^5	8	8	8	8	13u
	12	4×10^5	7	5	0	0	12u
	13	3×10^5	5	3	0	0	6u
	14	11×10^5	5	5	3	0	10u
	21	4×10^5	8	8	7	0	7u (9u)
	28	5×10^5	7	7	5	0	8 (8u)

* Figures indicate the interval (in weeks) between inoculation and the healing and disappearance of the nodule produced at the site of inoculation.

† u = ulceration of nodule.

‡ Figures in parentheses indicate diameter of nodules produced by the inoculation of a dose ten times as great as that indicated in the column "viable count."

to that described by Bloch (3) and confirmed by the present writers (28) with cultures of virulent tubercle bacilli.

If the over-all data are compared with the results of Suter and Dubos (37), it appears that the strain of BCG used in these experiments resembles most closely their Strain II, which was the least virulent of the three substrains which

they studied. Owing to possible differences in the susceptibility of the laboratory animals in the two series of experiments, no direct comparison is possible.

The immunogenicity of cultures of various ages: The degree of protection conferred upon mice by cultures of varying ages was tested by inoculating groups of 20 mice which belonged to the same batches as were used for the assessment of virulence of the cultures with 0.1 ml. of a 4-day-old culture of the virulent strain H37Rv about five weeks after they had been inoculated with BCG. Four batches of culture were used for the challenge inoculations, each being a freshly prepared Tween-albumin culture initiated from the same original suspension of bacilli and standardized by total count so that the inoculum contained approximately 6×10^6 bacterial units. Plate counts showed that in each case the viable count was within 10 per cent of the total count.

TABLE 10

A STATISTICAL COMPARISON OF THE SURVIVAL TIMES OF GROUPS OF MICE VACCINATED WITH BCG CULTURES OF VARIOUS AGES AND CHALLENGED WITH AN INTRAVENOUS INOCULATION OF VIRULENT TUBERCLE BACILLI

EXPERIMENT	MEAN DEATH TIME	LONGEST SURVIVAL	SHORTEST SURVIVAL	DIFFERENCE	t	P
	per cent					
I	25	59 (o)*	48 (28)	11	2.101	>0.05
	50	87 (7)	55 (28)	32	3.811	<0.01†
	75	81 (o)	74 (6)	7	0.806	>0.4
II	25	68 (7)	49 (13)	19	2.419	<0.05
Excluding 6-day	50	83 (7)	56 (21)	27	2.290	<0.05
and 11-day groups	75	80 (28)	70 (13)	10	1.403	>0.1

* 59 = average survival time of the indicated percentage of the group, in days; (o) = group inoculated with BCG culture 0 days old.

† P less than 0.01 indicates significant difference.

The mean death times in days (M.D.T.) for the first 25 per cent, the first 50 per cent, and the first 75 per cent of the mice to die in each group, and finally for the whole group when all had died, were determined for each vaccinated and control group. The ratios of the figures obtained for the vaccinated groups were compared with those obtained from the appropriate control groups. The mean death times of the first 5 and the first 10 animals to die in each control group did not differ greatly (19.6, 21.4, 21.8, and 21.6 days for the first 25 per cent; 20.8, 25.1, 24.7, and 23.2 days for the first 50 per cent). Nevertheless, the prolonged survival of a few mice in some of the groups introduced a greater variability in the mean death times for the other classifications ($M.D.T._{75\%}$ and $M.D.T._{100\%}$). Comparison of the $M.D.T._{50\%}$ of vaccinated and control mice was the most informative, and the ratios $\frac{M.D.T._{50\%}\text{ vaccinated mice}}{M.D.T._{50\%}\text{ control mice}}$ were determined and are included in tables 2 and 3. With two exceptions, the values varied between 2.3 and 3.6 and no obvious trend was apparent. In particular, the

values obtained for the very young cultures (twenty-four and forty-eight hours) and old cultures (twenty-one and twenty-eight days) were not significantly lower than other values. Cultures of these ages might have been expected to immunize mice less efficiently than cultures three to seven days old, from analogy with virulent tubercle bacilli (3), and, in the case of the older cultures, for a consideration of their somewhat lower virulence for the mouse and guinea pig (*see* previous section). The two exceptions (6-day-old and 11-day-old cultures, *Experiment II*, table 3) were affected by large numbers of mice which died on a particularly hot day (shade temperature 109° F.) without extensive signs of tuberculosis.

A statistical comparison of the longest and shortest survival times at M.D.T.$_{25\%}$, M.D.T.$_{50\%}$, and M.D.T.$_{75\%}$ in groups vaccinated with cultures of varying ages, with regard to the survival times with the corresponding control groups, is set out in table 10. The only significant difference observed was between the M.D.T.$_{50\%}$ at seven and twenty-eight days in *Experiment I*. In this instance, the 28-day-old culture appeared to be significantly less effective in protecting the mice against the challenge inoculation than was the 7-day-old culture. However, the differences in immunogenicity, as determined in these experiments, were not as great as might have been expected from a consideration of the virulence of the cultures of various ages. This may have been due to the very large dosage of organisms used for vaccinating the mice.

DISCUSSION

It was not until the introduction of Tween 80 by Dubos (1) that it became possible to study quantitatively the growth of *Mycobacteria in vitro* in a manner similar to that adopted for the study of other bacteria. In his review on the growth of bacterial cultures, Monod (10) insisted on the necessity for using aerated cultures for such studies. It has been observed (8, 40), however, that, if a Tween-albumin culture of mammalian tubercle bacilli is aerated either by continuous shaking or by a stream of sterile air, growth is granular and is much less in amount than is observed in cultures incubated in shallow layers without continuous agitation. In most of the present experiments, therefore, cultures were incubated in shallow layers (usually 30 ml. in the bottom of a 250 ml. Erlenmeyer flask, approximately 3.5 inches in diameter at the bottom) and agitated gently by hand once each day.

The only study comparable to the present one is that reported by Bretey and Imelik (41, 42), who studied the growth of *Mycobacterium avium* in the Tween-albumin medium by means of nephelometry, bacterial counts, and the study of films stained by the Ziehl-Neelsen method. The high degree of dispersion of avian tubercle bacilli is well known and was borne out by their studies. Starting with an inoculum which contained 98 per cent of isolated bacilli, they observed an increased aggregation which reached a maximum, with a large inoculum, on the third day, when the average number of bacilli in each clump was 7. Thereafter the bacilli separated, so that at eight days the culture again consisted almost entirely of single bacilli. With a smaller inoculum the highest clumping factor of 12 occurred on the seventh day and the culture did not become completely dis-

persed until the eleventh day. A similar trend, spread over a longer time interval consistent with the slower growth of mammalian tubercle bacilli, was observed in the present experiments, but the degree of dispersion was never as great. The trend was more apparent with BCG than with H37Rv. With both there was initially an increase in the degree of clumping, which remained at a constant level with an average of 6 to 8 bacilli in each bacterial unit until about the fourteenth day. Thereafter the clumps became smaller and single bacilli became more numerous, so that in the mature cultures (twenty-one and twenty-eight days old) the average number of bacilli per bacterial unit had fallen to approximately 2.

Several investigators have recently investigated the growth rate of tubercle bacilli in different culture media. Several methods have been used. The first was essentially the same as one of the methods employed in the present investigations, namely, photometric turbidity readings on cultures grown in the Tween-albumin medium (7, 8, 42, 43). Youmans (44) described a method involving micro-Kjeldahl nitrogen determinations, but later (45) supplanted this by a technically simpler method, depending upon the time required for the first visible appearance of growth after inoculation with a series of decimal dilution of tubercle bacilli. Imelik and Bretey (42) determined the generation time by bacterial counts as well as by photometry and, in the experiments herein reported, photometry, bacterial counts, and bacterial nitrogen determinations were all used. It is of interest to compare the generation times determined by different authors (table 11).

While there is considerable variation in the figures, it is apparent that, with mammalian tubercle bacilli grown in dispersed fashion in the Tween-albumin medium without continuous agitation, the generation time during the phase of exponential growth is usually between fifteen and twenty hours. The generation time of avian tubercle bacilli is considerably shorter. The unusually long generation times recorded when the rate of increase of bacterial nitrogen was used as the criterion (44) may be due partly to the more rapid growth with small inocula than with very large (45) or partly also to the method of assessment of bacterial growth. It was found in the present study that the generation time calculated from the bacterial nitrogen content was considerably longer in one experiment with BCG than the generation times calculated by other methods of measurement. Under certain conditions considerable reduction in the nitrogen content of bacilli may occur with only slight changes in the dry weight or opacity of the culture (48).

The only workers (7, 45) who have mentioned the lag phase in the growth of mammalian tubercle bacilli in complete media have used methods of investigation which have not permitted a comparison of the increase in cell mass with that in bacterial numbers. In the present experiments, a few observations were made on the lag phase. With H37Rv there appeared to be a definite lag phase. In comparable experiments, it was shorter when 3-day-old cultures were used as inocula than when 21-day-old cultures were used and was shorter in general when estimated by optical density than by bacterial numbers. With BCG there

appeared to be no lag when 3-day-old cultures were inoculated into fresh flasks of medium and rather long lag periods with 21-day-old inocula. The lag was again shorter when bacterial density rather than numbers was measured. These observations emphasize the similarity between the growth cycles of the pathogenic *Mycobacteria* and those of other bacteria.

TABLE 11

THE GENERATION TIMES OF TUBERCLE BACILLI GROWING IN LIQUID MEDIA, DETERMINED BY A VARIETY OF METHODS

STRAIN OF MYCOBACTERIUM	CULTURE MEDIUM	TECHNIQUE	GENERATION TIME	REFERENCE
			hours	
H37Rv	Modified Proskauer and Beck	Bacterial nitrogen	37–89*	(44)
H37Rv	Modified Proskauer and Beck	First appearance of growth	19.9	(45)
	Modified Proskauer and Beck plus crystalline bovine albumin		17.3	
	Modified Proskauer and Beck plus bovine serum		14.4	
H37Rv and 13 other strains	Modified Proskauer and Beck plus bovine serum	First appearance of growth	13.2–15.7	(46)
H37RvR	Tween-albumin	Photometric	21.1	(47)
H37Rv	Modified Tween-albumin (0.025 mg. NH_4Cl per ml., 10 mg. glucose per ml.)	Photometric	27–32*	(8)
H37Rv	Tween-albumin	Photometric	16–19*	(28)
	Tween-albumin	Total bacterial units	18–23*	
BCG	Tween-albumin	Photometric	17–23* (usually 20–23)	this paper
	Tween-albumin	Total bacilli	15–32* (usually 20–23)	
	Tween-albumin	Bacterial nitrogen	24	
M. avium	Tween-albumin	Photometric†	12	
	Tween-albumin	Total bacilli	6 and 7.6	(42)

* Results for different experiments varied within the limits shown.

† This value differs from that given by the authors of the paper (30–40 hours). It has been determined by converting their data to $\log_2$ values and replotting.

The observations of Fisher and Kirchheimer (13, 14) on the occurrence of a stage of arithmetic linear growth in cultures of tubercle bacilli in Tween-albumin media are confirmed by the present experiments. Further, it is apparent from the experiments on BCG that a phase of arithmetic linear growth can be recognized whether one uses the optical density readings, the number of bacilli, or the total nitrogen estimations. Arithmetic linear growth usually commenced earlier with the bacterial count than with the optical density. The occurrence of arithmetic linear growth in the bacterial nitrogen and in the total counts and the

close correspondence between total and viable counts throughout this stage of the growth cycle exclude one of the suggested explanations (13), namely, coincident exponential growth and autolysis.

Imelik and Bretey (42) comment upon the continued linear increase in optical density (on an arithmetic scale) after the stationary phase has been reached in the total count. If both sets of readings given in their paper are plotted arithmetically or semilogarithmically, this difference disappears and it is apparent that, like mammalian tubercle bacilli, the growth of *M. avium* in Tween-albumin medium is characterized by a stage of arithmetic linear growth which commences slightly later when estimated from the bacterial count rather than from the optical density.

Tubercle bacilli undergo marked elongation in the first day or so after introduction into fresh culture medium. This elongation, and the subsequent decrease in size, was greater in BCG than in H37Rv, and comments of other authors indicate that pronounced differences occur between different strains of mammalian tubercle bacilli. Mackaness and Smith (49), for example, noted that their strain "Branch" assumed a cocco-bacillary form in 10-day-old Tween-albumin cultures, whereas H37Rv never became as short. Similar differences in the size of two substrains of BCG from different sources have been demonstrated by van Diense and his collaborators. The difference was apparently characteristic of the substrains and independent of the culture medium used, at least for the first four passages. It is apparent from the results in the present study that, when comparing the size of tubercle bacilli, the culture medium, the size and age of the inoculum, and the age of the culture must be carefully defined.

Nuclear staining of *Mycobacteria* is not as satisfactory as that of many other bacteria, but it appeared from one experiment that changes in the nuclei preceded by an hour or so the elongation of the bacilli, and thereafter proceeded so that there was a rough parallel between the numbers of nuclei and the length of the bacilli. No observations were made of the relative proportions of undifferentiated, intermediate, and differentiated forms; but there appears to be a close parallel between the nuclear events described for *E. coli* (51) and those occurring with the *Mycobacteria*, except that the time scales are quite different.

In many investigations, suspensions of *Mycobacteria* are standardized by the measurement of their optical density. For most of the experimental problems of bacterial chemistry and metabolism, where the important variable is the bacterial density, this method is the most satisfactory. If the test depends upon the number of viable cells, as, for example, in virulence tests or genetic studies, this method of standardization can introduce considerable errors, due to the varying size of bacilli at different stages of growth. This is apparent from the growth curves and also in the data shown in table 1 of the present report and the immediately preceding paper. Added to this variability within strains is the variability between strains already mentioned. The close correspondence of total and viable counts during the periods and under the conditions of experimentation employed in these experiments renders it possible to determine the total number of bacterial units (which in these cultures corresponds closely with

the viable count) by direct counting in a Petroff-Hausser chamber—a simple and relatively accurate technique when the bacterial concentration is high enough. If it is necessary to know the total number of bacilli, differential counts of the numbers of bacilli per bacterial unit will allow the estimation of a clumping factor. In genetic studies the significant count may be that of the number of bacterial nuclei. The cytologic studies reported here indicate, not only that uninucleate bacilli are rare, but that the average nuclear count varies considerably at different stages of the growth cycle. As a very rough approximation, it may be stated that, with a 7-day-old Tween-albumin culture of H37Rv, the total count of the number of bacterial units should be multiplied by 3 or 4 to give the "total bacilli" count and again by 3 or 4 to give an estimate of the number of nuclei. The corresponding figures for BCG are 5 to 7 and 2 to 3. These figures vary with inocula of different sizes and different types of media so

TABLE 12

CORRELATION OF THE AGE OF TWEEN-ALBUMIN CULTURES WITH VARIOUS MORPHOLOGIC AND PHYSIOLOGIC CHARACTERS

AGE OF CULTURE	VIRULENCE FOR MICE	STAGE OF GROWTH CYCLE	CLUMPING FACTOR	AVERAGE LENGTH OF BACILLI	AVERAGE NUMBER OF NUCLEI PER BACILLUS	NEUTRAL RED REACTION	METHYLENE BLUE DECOLORIZATION*
				μ			
18–24 hours	Low	Lag phase	4–5	3.5	4.6	Weakly positive	Negative
3 days	Very high	Exponential phase	5–6	2.4	3.0	Strongly positive	Negative
7 days	High	Retardation phase	3–4	2.5	3.6	Strongly positive	Negative
21 days	Low	Stationary phase	2–3	2.4	2.6	Strongly positive	Positive

* Data from Bloch (3, 52).

that a direct estimation under the conditions of experimentation used should be carried out when exact information is required.

The results reported in the first paper confirm Bloch's (3) observations upon the effect of the age of the culture upon the virulence of tubercle bacilli. They offer no information upon the reasons for these differences in virulence. Engbaek (11), observing the great delay in growth on solid medium of inocula from 3-week-old Tween-albumin cultures of H37Rv, compared with 3-day-old cultures, has suggested that the differences in virulence may be related to this. In the experiments of the present writers with H37Rv, the lag time with older bacilli appeared to be only slightly greater than that with younger bacilli and both were relatively short. However, the differences between the media used for cultivation of the inoculum and its subsequent inoculation were much less than in Engbaek's experiments. The differences between culture media and host tissues are probably still greater, and the lag time might also be longer. It would not be difficult to test

this factor experimentally. The differences in lag time of young (3-day-old) and older (21-day-old) cultures of BCG were much greater than was the experience with H37Rv. In table 12 are listed the different morphologic and physiologic features of cultures of H37Rv associated with varying degrees of virulence for mice.

No attempt was made to determine the content of Bloch's (53) "cord factor" in cultures of different ages, but it is apparent that cultures of tubercle bacilli incubated for different periods vary in a large number of characters. It may be that one or more of these characters is important in determining the virulence of the cultures, perhaps in association with the development of the bacillary lipids (54), including the cord factor.

The virulence of the BCG cultures, in so far as it could be assessed by the methods used, appeared to be relatively stable until the cultures became mature (21 days old or more), when it dropped somewhat. Nevertheless, the degree of protection against challenge with H37Rv, conferred upon mice previously inoculated with BCG cultures with the same content of viable units but from cultures of different ages, appeared to be unaffected by the age of the culture. Vaccinated mice lived, on the average, two to three times as long as the control animals.

SUMMARY

The growth of BCG in Tween-albumin liquid medium was studied at frequent intervals for the first two days and at longer intervals thereafter for a month.

The first change observed after the inoculation of the bacilli into the flask was an elongation of the bacilli, which occurred between the eighth and the twenty-fourth hours. The bacilli remained elongated throughout the exponential phase, and the nuclear count was correspondingly high. After the end of the exponential phase there was a gradual diminution in the length of the bacilli and in the average numbers of nuclei per bacillus, and by the end of the third week both reached quite low values.

The optical density of the inoculated flasks began to increase within a few hours of inoculation with the younger inocula, and the bacterial count rose shortly afterwards. With large inocula of 21-day-old "starter" cultures the increase in opacity, and especially in bacterial numbers, was greatly delayed. After a short period of acceleration the increase in both properties became exponential. The generation time of BCG during the exponential phase was approximately twenty hours. After the period of exponential growth there was a phase lasting about seven days, during which growth was linear when plotted arithmetically. Determinations of the nitrogen content of the culture showed that this also passed through stages of exponential and arithmetic linear growth, although they were not as clearly defined as with the other properties of the culture.

During the growth of the culture the proportion of clumps containing several bacilli increased for the first four days, was maintained at a roughly constant level during the next six days, and then fell so that cultures aged fourteen days or more consisted predominantly of single bacilli or clumps of two or three bacilli.

The virulence of the BCG cultures appeared to be somewhat lower with cultures aged twenty-one days and twenty-eight days than with younger cultures,

but age did not appear to affect the immunogenicity of the cultures under the conditions of the experiments. As far as could be judged, the BCG strain used in the present investigations was of rather low virulence. Mice inoculated intravenously with large doses of BCG five weeks before challenge inoculation with H37Rv lived two to three times as long as the unvaccinated controls.

SUMARIO

El Desarrollo de los Bacilos Tuberculosos de Mamífero en Medio Líquido de Tween-Albúmina: II. Observaciones sobre la Proporción de Microbios Viables, la Citología, la Virulencia y la Antigenicidad en Diversos Períodos del Desarrollo de BCG

El desarrollo de BCG en el medio líquido de Tween-albúmina fué estudiado a plazos frecuentes durante los primeros dos días y más largos a partir de entonces durante un mes.

La primera alteración observada después de la introducción de los bacilos en el balón fué un alargamiento de los mismos, que tuvo lugar entre la octava y la vigésimacuarta horas. Los bacilos permanecieron alargados durante toda la fase exponencial y la numeración nuclear fué alta en forma correspondiente. Después de terminar la fase exponencial, hubo una disminución gradual del largo de los bacilos y del número medio de núcleos por bacilo, alcanzando ambos cifras bastante bajas para el final de la tercera semana.

La densidad óptica de los balones inoculados comenzó a aumentar a las pocas horas de la inoculación con los bacilos más jóvenes y la numeración bacteriana se elevó poco después. Con los inóculos grandes de cultivos de veintiún días, se demoró considerablemente el aumento de la opacidad y, sobre todo, del número de bacterias. Al cabo de un breve período de aceleración, el aumento de ambas propiedades se volvió exponencial. El tiempo de generación de BCG durante la fase exponencial fué de unas veinte horas. Después del período de crecimiento exponencial, hubo una fase, que duró unos siete días, durante la cual el desarrollo resultó lineal al ser computado aritméticamente. Las determinaciones del contenido de nitrógeno del cultivo revelaron que también mostraba períodos de desarrollo exponencial y lineal aritmético, aunque no tan bien definidos como los de las otras propiedades del cultivo.

Durante el desarrollo del cultivo la proporción de grumos que contenían bacilos aumentó durante los primeros cuatro días, se mantuvo a un nivel más o menos constante durante los seis días siguientes, y luego descendió, de modo que los cultivos de catorce días o más de edad constaban predominantemente de bacilos aislados o de grupos de dos o tres bacilos.

La virulencia de los cultivos de BCG pareció ser algo menor en los de veintiún y veintiocho días que en los más jóvenes, pero la edad no pareció afectar la inmunigenicidad de los mismos, en las condiciones de los experimentos. En lo que pudo apreciarse, la cepa de BCG usada en las investigaciones actuales poseía virulencia algo baja. Los ratones inoculados intravenosamente con grandes dosis de BCG cinco semanas antes de la inoculación provocativa con H37Rv vivieron el doble o el triple que los testigos no vacunados.

RESUME

La croissance des bacilles tuberculeux de mammifères dans un milieu liquide. 2. Observations sur la proportion d'organismes viables, la cytologie, la virulence et les propriétés antigènes du BCG à différents stades de la croissance

La croissance du BCG dans le milieu liquide Tween-albumine a été étudiée à intervalles fréquents pendant les deux premiers jours et à intervalles plus longs ensuite pendant un mois.

Le premier changement observé après l'ensemencement du bacille dans le ballon était un allongement des bacilles, qui se produisait entre la huitième et la vingt-quatrième heure. Les bacilles demeuraient allongés pendant toute la phase exponentielle et la numération nucléaire était proportionnellement élevée. Lorsque la phase exponentielle était terminée,

il se produisait une diminution graduelle de la longueur des bacilles et du nombre moyen des noyaux par bacille; à la fin de la troisième semaine ces deux valeurs atteignaient un niveau très bas.

La densité optique des ballons ensemencés commençait à s'accroître dans les quelques heures qui suivaient l'inoculation avec l'inoculum plus jeune et le nombre des bacilles s'élevait bientôt après. Avec un gros inoculum de cultures initiales de vingt-et-un jours, l'accroissement du degré d'opacité et particulièrement celui du nombre des bactéries subissait un retard considérable. Après une courte période d'accélération, l'augmentation de ces deux propriétés devenait exponentielle. Le temps de reproduction du BCG au cours de la phase exponentielle était d'environ vingt heures. A la suite de la période exponentielle de la croissance, il y avait une phase qui durait environ sept jours, au cours de laquelle on observait une croissance linéaire arithmétique. Les analyses de la teneur en nitrogène de la culture montraient que celle-ci passait également par des phases de croissance exponentielle et des phases linéaires arithmétiques; ces phases étaient toutefois moins nettement définies que celles observées pour les autres propriétés de la culture.

Au cours de la croissance de la culture, la proportion des agglomérats contenant plusieurs bacilles augmentait pendant les quatre premiers jours. Cette proportion était sensiblement constante pendant les six jours suivants et s'abaissait brusquement ensuite, de telle sorte que les cultures âgées de quatorze jours ou plus consistaient surtout en bacilles séparés ou en agglomérats de deux ou trois bacilles.

La virulence des cultures de BCG paraissait légèrement plus réduite en ce qui concerne les cultures âgées de vingt-et-un et de vingt-huit jours que lorsqu'il s'agissait de cultures plus jeunes. Toutefois, dans les conditions de l'expérience, l'âge ne paraissait pas affecter les propriétés immunisantes des cultures. Autant qu'on peut en juger, la souche BCG utilisée dans ces investigations était d'une virulence relativement basse. Les souris étaient soumises à l'inoculation intraveineuse de fortes doses de BCG cinq semaines avant l'inoculation expérimentale avec le H37Rv survivaient deux ou trois fois plus longremps que les témoins non-vaccinés.

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ELECTROPHORETIC STUDIES OF THE SERUM PROTEINS IN TUBERCULOSIS[1]

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INTRODUCTION

Electrophoretic studies of the serum protein changes in human tuberculosis have been reported by Seibert, Seibert, Atno, and Campbell (1), by Ewerbeck (2), and by Knüchel and Kienle (3). These studies showed that as the disease progressed, there were increases in the alpha and gamma globulins with corresponding decreases in the albumin, and it was suggested that the increase in gamma globulin was due to antibody formation. No attempt was made, however, to isolate and identify antibodies in the gamma globulin fraction, although Emmart and Seibert (4) showed that the fraction possessed tuberculostatic properties. Recently, Small (5) has investigated the serum gamma globulins in pulmonary tuberculosis, using a turbidimetric technique developed by Kunkel (6). The results did not agree with the findings of Seibert and co-workers (1), and it was found that there was a good deal of overlapping of values in minimal, moderately advanced, and far advanced cases.

The present work was carried out in order to confirm the findings of Seibert and her co-workers (1), and the fraction of tuberculous human serum containing antibodies was identified in order to correlate the observed changes with antibody formation. In addition, the serum protein changes which occur during the treatment of pulmonary tuberculosis were studied.

METHODS

Cases of tuberculosis were selected by one of us (C. N. I.) and classified as follows:

Minimal pulmonary tuberculosis: These patients had unilateral lesions and no loss of weight or other symptoms.

Advanced pulmonary tuberculosis: These patients had acute bilateral exudative phthisis as defined for the purposes of the Medical Research Council streptomycin trials (7).

Far advanced pulmonary tuberculosis: The patients had extensive bilateral lesions and were in a poor clinical condition; the prognosis was bad.

Advanced nonpulmonary tuberculosis: All of these patients had active lesions in the spine and, in addition, 3 had kidney lesions. Quiescent chest lesions were present in 2 patients. This group was less homogeneous than the others.

Normal serum was obtained from healthy Mantoux-positive adults.

Blood was withdrawn by venepuncture into a sterile bottle, allowed to clot at room temperature, and then the serum was removed aseptically and re-centrifuged. Serum was often

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cloudy, due to the presence of lipid material. This was removed by centrifugation (15,000 r.p.m. at 0°C.), and the clear serum was sucked from under the lipid which rose to the surface.

Electrophoretic Technique

All of the analyses of serum were performed in barbiturate (Veronal) buffer at pH 8.6, and ionic strength 0.1 (8). Each serum sample was diluted with twice its volume of barbiturate buffer, dialyzed against the same buffer at 1°C. for forty-eight hours, and clarified by centrifugation. Then the serum was examined in a Tiselius electrophoresis apparatus fitted with a cylindrical lens, schlieren optical system (9).

Electrophoretic patterns were enlarged four to five times and the outlines traced. These diagrams were resolved into components by the method of Svedberg and Pedersen (10), and the area due to each component was measured with a planimeter. The ratio of the area of a given peak to the sum of the areas of all the peaks, neglecting the area of the delta or epsilon anomaly, was taken as the relative concentration of the component responsible for the peak. In the analysis of a sample, the relative percentage composition was determined from patterns recorded at intervals during the experiment and the values were averaged. In the majority of the electrophoretic patterns recorded, there was a large anomaly present

TABLE 1

CONTENTS OF SERUM FRACTIONS ISOLATED ELECTROPHORETICALLY

FRACTION	POSITION IN CELL	CONTENTS
I	Top ascending section	Albumin, alpha, and beta globulins in reduced concentration. No gamma globulin.
III	Bottom section	Whole serum at its original concentration.
IV	Lower descending section	Albumin, alpha, beta, and gamma globulins in slightly different proportions to whole serum and at a slightly reduced concentration.
V	Top descending section	Gamma globulin, together with a trace of beta globulin, in reduced concentration.

in the descending beta globulin boundary, and this made interpretation of the descending patterns difficult. Therefore, all of the data recorded in this paper were computed by using only the ascending patterns.

Electrophoretic Isolation of Serum Components

Undiluted serum was dialyzed against a buffer solution at 1°C. for forty-eight hours and then examined electrophoretically. The electrophoresis was continued until the serum components were completely resolved and extended throughout the cell. The current was then stopped and serum fractions were isolated from the electrophoresis cell. The contents of each fraction are listed in table 1.

Initially, the fractionations were performed in 0.15 M. sodium chloride buffered at pH 7.6 with an 0.02 M. sodium phosphate buffer. Separation of the serum components, especially albumin and alpha-1 globulin, was incomplete in this buffer, however, and later experiments were performed, therefore, using the barbiturate (Veronal) buffer at pH 8.6 and ionic strength 0.1. Resolution of the serum components was more complete in this buffer and it was possible to isolate the gamma globulin fraction (Fraction V) free of beta globulin. Barbiturate buffer at pH 8.6 is anticomplementary and, therefore, all fractions isolated in this buffer were re-dialyzed against a sodium chloride-barbiturate buffer at pH 7.3 before testing for serologic activity by the complement fixation test. The sodium chloride-barbiturate buffer consisted of 0.15 M. sodium chloride, containing a dilute barbiturate buffer at pH 7.3 (11).

Serologic Activity, Percentage Composition, and Nitrogen Content

The serologic activity, protein concentration, and relative percentage composition of each fraction were determined as follows:

Relative percentage composition of each fraction was determined by the method of Erickson and co-workers (12).

Nitrogen content was determined by micro-Kjeldahl, using Markham's apparatus (13).

Serologic activity was assessed by the complement fixation test.

The serum, or serum fraction, was inactivated by heating at 56°C. for thirty minutes. Then dilutions of each fraction were incubated at 37°C. for thirty minutes with a constant volume of antigen and in the presence of two minimum hemolytic doses of complement (guinea pig). The hemolytic system was then added and the mixtures reincubated at 37°C. for thirty minutes. The results were read immediately after the second incubation. The antigen used was a suspension of *M. tuberculosis* which had been extracted with urea (14).

TABLE 2

ELECTROPHORETIC ANALYSES OF TUBERCULOUS HUMAN SERUM

Means and the Significance of Their Deviations from the Normal Means

DIAGNOSIS	NUMBER OF CASES	RELATIVE PERCENTAGE COMPOSITION										ALBUMIN/GLOBULIN RATIO	
		Albumin		Globulins									
				Alpha-1		Alpha-2		Beta		Gamma			
		Per Cent	*P*	Per Cent	*P*	Per Cent	*P*	Per Cent	*P*	Per Cent	*P*	Per Cent	*P*
Normal adult	5	55.1		4.0		8.8		14.8		17.2		1.23	
Pulmonary tuberculosis													
Minimal	6	53.1	>0.1	4.6	>0.1	10.3	>0.1	15.6	>0.1	16.3	>0.1	1.13	>0.1
Advanced	7	39.0	<0.001	7.8	<0.05	14.3	0.05	15.8	>0.1	23.1	<0.001	0.64	<0.001
Far advanced or terminal	7	39.6	<0.001	8.3	<0.05	15.3	<0.001	12.8	>0.05	24.0	<0.05	0.66	<0.001
Nonpulmonary tuberculosis advanced	8	47.4	<0.05	6.2	0.05	12.6	<0.001	15.0	>0.1	18.8	>0.1	0.90	0.05

P is the probability corresponding to the calculated value of Fisher's *t*.

RESULTS

Serum Protein Changes in Tuberculosis

The results of the electrophoretic analyses of tuberculous human serum are summarized in table 2 which shows for each type of case the mean and the significance of the deviation of the mean from the normal mean. Figure 1 shows typical electrophoretic patterns of serum obtained at different stages of the disease.

No significant changes were detected in the serum from patients with minimal pulmonary tuberculosis. However, as the disease became more severe, there were increases in the alpha-1, alpha-2, and gamma globulins with corresponding decreases in albumin. In severely ill patients, there were further increases in the alpha-1 and alpha-2 globulins and a slight decrease in the beta globulin. The alpha-1 and alpha-2 globulin levels in serum from advanced and far advanced cases fluctuated considerably and there was a wide overlapping of values. Thus, in some cases, the alpha globulin levels were not very different from the normal values, while in others there was a threefold increase. The largest increases in

alpha globulin were detected in serum from patients with far advanced disease whose general condition was poor. The most consistent change detected in the advanced stages of the disease was the increase in gamma globulin.

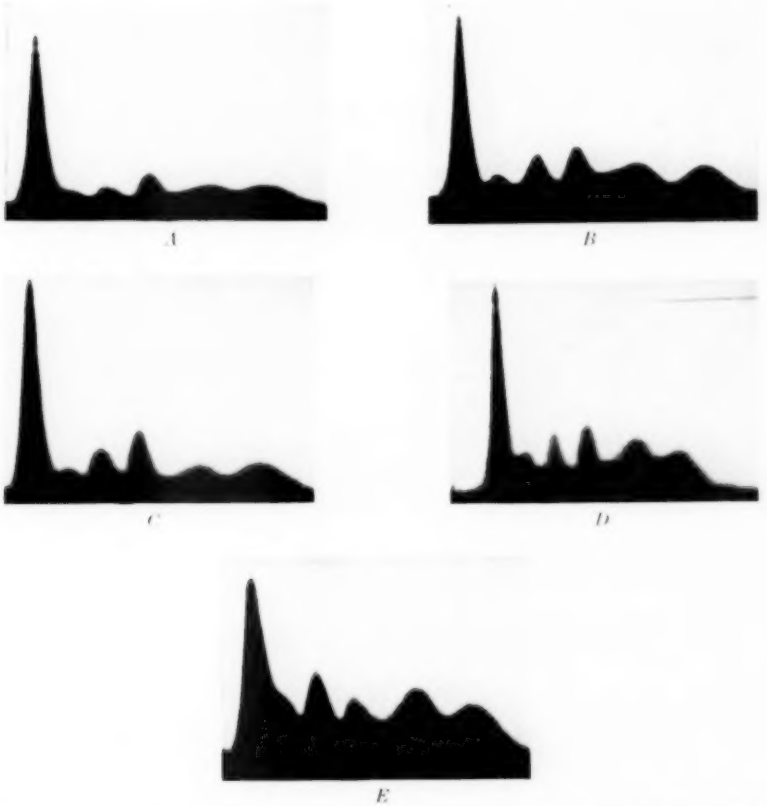

FIG. 1. Electrophoretic diagrams of human serum examined in Veronal buffer, pH 8.6, ionic strength 0.1. Only ascending patterns are shown.

A. Normal
B. Advanced nonpulmonary tuberculosis
C. Minimal pulmonary tuberculosis
D. Moderately advanced pulmonary tuberculosis
E. Far advanced pulmonary tuberculosis

In the sera from patients with tuberculosis of the bones, the serum protein levels were considerably scattered, and, therefore, it was not possible to draw reliable conclusions from the small number of these cases studied. The only definite changes were rises in the alpha-1 and alpha-2 globulins, with corresponding decreases in albumin.

Serum Protein Changes Which Occurred During the Treatment of Tuberculous Patients

The 4 patients studied had advanced bilateral pulmonary tuberculosis and had been selected for treatment in the Medical Research Council's clinical trials of streptomycin and *p*-aminosalicylic acid (7). A sample of the patient's serum was analyzed electrophoretically before the treatment began and further samples

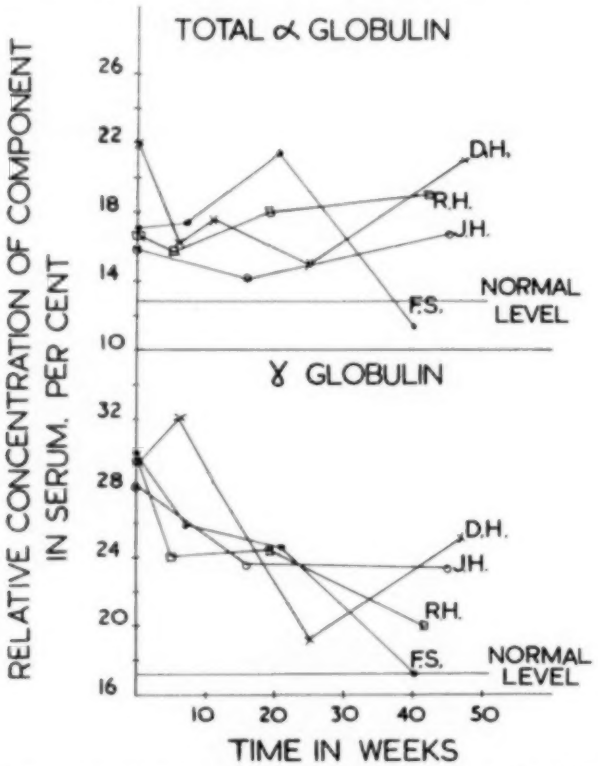

FIG. 2. Changes observed in the gamma and total alpha (alpha-1 plus alpha-2) globulin levels during the treatment of patients with pulmonary tuberculosis.

were examined at intervals during and after treatment. The results are summarized in figure 2, which shows the changes observed in the gamma and total alpha (alpha-1 plus alpha-2) globulin levels during the courses of treatment. Although the results are not numerous enough to permit statistical analysis, they do show that, as recovery occurred, the gamma globulins gradually decreased toward the normal level. In one patient (D. H.), a sudden increase in gamma globulin coincided with deterioration in the patient's condition. Changes in the alpha globulins were less definite, and in only one case (F. S.) did the alpha

globulin level decrease to the normal level. None of the sera examined had abnormal beta globulin levels and no change was detected in this component during the treatments.

Identification of Antibodies in Tuberculous Human Serum

(in collaboration with Dr. G. A. Gilbert)

At first an attempt was made to identify the fraction of tuberculous human serum containing antibodies, using the method devised by Tiselius and Kabat (15).

In this method, a sample of serum is analyzed electrophoretically while another is treated with the antigen to remove the antibody and is then analyzed under exactly the same conditions as the original sample. The measured area of the albumin peak of the treated sample is adjusted so that the areas of the albumin peak in the original and treated samples are the same (the albumin peak is unlikely to be affected by the removal of antibody). Then, the areas of the other components in the treated sample are corrected by multiplying the measured areas by the ratio of the measured areas of the albumin peaks in the original and treated samples. The removal of antibody is indicated by a decrease in area of one of the components.

TABLE 3

ABSORPTION OF TUBERCULOUS HUMAN SERUM WITH M. TUBERCULOSIS

SERUM	COMPOSITION									
	Albumin		Globulins							
			Alpha-1		Alpha-2		Beta		Gamma	
	Cm.²	Per Cent	Cm.²	Per Cent	Cm.²	Per Cent	Cm.²	Per Cent	Cm.²	Per Cent
Normal adult (mean of 5 samples)		55.1		4.0		8.8		14.8		17.2
1) Abnormal (J. H.)										
Original serum	500	40.8	57	4.7	140	11.4	185	15.1	342	27.9
Treated sample	500	43.4	48	4.2	118	10.2	146	12.7	340	29.5
Observed change	—	+2.6	−9	−0.5	−22	−1.2	−39	−2.4	−2	+1.6
2) Abnormal (E)										
Original serum	367	41.8	64	7.3	117	13.3	125	14.2	206	23.4
Treated sample	367	45.3	43	5.3	110	13.6	108	13.3	183	22.6
Observed change	—	+3.5	−21	−2.0	−7	+0.3	−17	−0.9	−23	−0.8

Two specimens of serum from patients with advanced bilateral pulmonary tuberculosis were treated in this manner. Both specimens had increased alpha-2 and gamma globulins, the gamma globulin increase being the predominant change. Absorption of these sera did not produce any significant change in the electrophoretic patterns (table 3). The sera were absorbed twice with a suspension of living virulent *M. tuberculosis*.

Since the above experiments showed that absorption did not remove detectable amounts of antibody from the sera, an attempt was made to isolate the serum fraction containing antibodies. Altogether, five specimens of serum from patients with active bilateral pulmonary tuberculosis were fractionated electrophoretically, and the serologic activity, protein concentration, and relative per-

TABLE 4

COMPOSITION AND SEROLOGIC ACTIVITY OF ELECTROPHORETICALLY ISOLATED FRACTIONS OF TUBERCULOUS HUMAN SERUM

PATIENT	SERUM FRACTION	COMPOSITION (MG. PER ML.)					DILUTION TITER OF ISOLATED SERUM FRACTIONS
		Albumin	Globulins				
			Alpha-1	Alpha-2	Beta	Gamma	
			α_1 + α_2				
T.	I	19.6	1.6		1.0	0	0
	III	34.5	4.9		7.6	11.6	1/2
	IV	14.9	4.9		9.0	13.8	1/2
	V	0	0	0	0.2	3.7	1/1
M.	I	23.3	2.0	4.8	1.4	0	0
	III	—	—	—	—	—	1/8
	IV	—	—	—	—	—	1/8
	V	0	0	0	0	4.4	1/5
A.	I	20.8	2.8	3.8	1.2	0	0
	III	27.1	3.6	9.2	7.1	9.3	1/4
	IV	—	—	—	—	—	1/2
	V	0	0	0	0	2.8	1/2
A. B.	I	11.1	1.6	2.8	1.9	0	0
	III	29.6	3.2	8.3	8.7	6.3	1/40
	IV	0	1.8	2.1	3.5	2.9	1/10
	V	0	0	0	0	2.4	1/10
			α_1 + α_2				
G. S.	I	24.5	4.4		2.1	0	0
	III	31.0	6.9		6.5	8.8	1/20
	IV	4.7	4.4		6.8	8.8	1/10
	V	0	0		0	3.9	1/8

centage composition of the fractions were determined. The results summarized in table 4 show that the complement-fixing antibodies in the serum are contained in the gamma globulin fraction. All fractions containing gamma globulin showed some activity, while the fraction devoid of gamma globulin (Fraction I) was inactive. The fraction containing mainly gamma globulin, together with a trace of beta globulin (Fraction V), was active. It is unlikely that the activity of this fraction was due to beta globulin, because the analyses of the fractions showed

that, apart from gamma globulin, any other component present in this fraction was present in a much greater concentration in Fraction I, and this was inactive.

DISCUSSION

The data obtained in the study of the serum protein changes which occur in pulmonary tuberculosis show that, as the disease progresses, there are increases in the alpha-1, alpha-2, and gamma globulins, together with corresponding decreases in albumin. In severely ill patients, there are further increases in the alpha-1 and alpha-2 globulins. These results confirm the findings of Seibert and associates (1) although a good deal of overlapping of values was found between moderately and far advanced cases.

A study of the serum protein changes which occurred during the treatment of 4 tuberculous patients with streptomycin and *p*-aminosalicylic acid indicated that, as the patients recovered, there was a gradual decrease in gamma globulin toward the normal value. Similar results have been reported by Ewerbeck (2) in a study of the serum protein changes occurring during the course of miliary tuberculosis and tuberculous meningitis. In a study of the plasma protein changes which occurred during the treatment of tuberculous patients with a thiosemicarbazone, Knüchel and Kienle (3) detected a decrease in the alpha globulin and an increase in gamma globulin. They suggested that the increase in gamma globulin was due to antibody formation but, in view of the toxic effects of the thiosemicarbazone on the liver and the results reported in the present paper, this statement needs further examination.

As increases in the alpha and gamma globulins were demonstrated in tuberculous human serum, an attempt was made to determine whether these changes were due to antibody formation. At first absorption experiments were tried, but no significant changes were detected when tuberculous human serum with elevated alpha and gamma globulin levels was absorbed with a suspension of living virulent *M. tuberculosis*. A suspension of living virulent organisms is probably the best antigen for the complement-fixation test and, therefore, the results suggest that the amount of antibody is so small that its removal does not alter appreciably the electrophoretic pattern of the serum. If this is so, then the greater part of the increased gamma globulin found in tuberculous human serum is not antibody.

A study of the complement-fixing activity of electrophoretically isolated serum fractions revealed that the complement-fixing antibodies were contained in the gamma globulin fraction.

SUMMARY

In pulmonary tuberculosis, increases in the alpha-1, alpha-2, and gamma globulins occur in the serum as the disease progresses. These changes are accompanied by corresponding decreases in albumin. Increases in the alpha-1 and alpha-2 globulins, together with corresponding decreases in albumin, were detected in the serum from patients with orthopedic and renal tuberculosis.

A study of the serum protein changes which occur during the treatment of

pulmonary tuberculosis has shown that the gamma globulin level decreases toward the normal level as the patient recovers.

The complement-fixing antibodies in the serum of patients with advanced pulmonary tuberculosis have been shown to be contained in the gamma globulin fraction.

Absorption of tuberculous human serum with *M. tuberculosis* did not produce any significant changes in the electrophoretic patterns. It is probable that most of the increased gamma globulin detected in the serum is not antibody.

SUMARIO

Estudios Electroforéticos de las Seroalbúminas en la Tuberculosis

En la tuberculosis pulmonar, obsérvanse aumentos de las globulinas alfa-1, alfa-2 y gamma en el suero sanguíneo a medida que avanza la enfermedad. Esas alteraciones van acompañadas de disminuciones correspondientes de la albúmina. En el suero de enfermos que tenían tuberculosis ortopédica y renal, descubriéronse aumentos de las globulinas alfa-1 y alfa-2 con disminuciones correspondientes de la albúmina.

El estudio de las alteraciones que tienen lugar en la proteína sérica durante el tratamiento de la tuberculosis pulmonar demostró que, a medida que se restablece el enfermo, la cifra de globulina gamma disminuye hacia la fórmula normal.

Según se demostró, los anticuerpos fijadores del complemento en el suero de los enfermos que tenían tuberculosis pulmonar avanzada estaban contenidos en la fracción gamma de la globulina.

La absorción con *M. tuberculosis* del suero humano tuberculoso no produjo mayores alteraciones en los patrones electroforéticos. Es probable que la mayor parte de la globulina gamma humana aumentada descubierta en el suero no represente anticuerpos.

RESUME

Etudes électrophorétiques des protéines sériques dans la tuberculose

Dans la tuberculose pulmonaire, à mesure que la maladie progresse, il se produit dans le sérum une augmentation des globulines alpha-1, alpha-2 et gamma. Ces modifications s'accompagnent d'une diminution correspondante de l'albumine. L'augmentation des globulines alpha-1 et alpha-2 avec réduction correspondante de l'albumine sérique a été décelée chez des malades affectés de tuberculose rénale ou osseuse.

Une étude des modifications de la protéine sérique survenant au cours du traitement de la tuberculose pulmonaire a montré que la concentration de la globuline gamma diminue et tend à se rapprocher de la normale à mesure que l'état du malade s'améliore. Il a été démontré que les anticorps fixateurs du complément du sérum de malades présentant une tuberculose pulmonaire avancée sont contenus dans la fraction globuline gamma.

L'absorption du sérum tuberculeux humain avec *M tuberculosis* ne produisait aucune modification appréciable des tracés électrophorétiques. Il y a lieu de penser que la plus grande partie de cette globuline gamma augmentée, décelée dans ce sérum n'agit pas comme anticorps.

Acknowledgment

We wish to thank Professor M. Stacey and Dr. G. A. Gilbert of the Department of Chemistry, University of Birmingham, for their advice, and Dr. J. E. Geddes, Chief Clinical Tuberculosis Officer, City of Birmingham, for the sera. The Medical Research Council provided both authors with personal grants and also a grant for expenses.

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ACUTE TUBERCULOUS PNEUMONIA IN THE NEGRO[1,2]

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INTRODUCTION

Until recently the treatment of acute massive pneumonic tuberculosis has been disappointing, particularly in Negroes. Of 32 Negroes treated by Shields (4) before streptomycin became available, 25 died and 3 were hopelessly ill at the time of his report; at the same time he reported a mortality of 40 per cent in a series of 20 white patients. Large tuberculous consolidations are far more common in Negro than in white patients. In 1952, 15 Negroes and 4 white patients were admitted with massive tuberculous pneumonia to the Tulane University Service at Charity Hospital.

Acute massive tuberculous pneumonia has a characteristic clinical pattern. The onset is fairly acute and the duration of symptoms is usually less than one month when the patient is first seen. Fever and constitutional symptoms are present together with productive cough. Definite physical findings of pulmonary consolidation are always found. Often these patients are admitted to general medical wards but the duration of symptoms should arouse early suspicion of tuberculosis. The chest roentgenogram portrays either a large area of consolidation involving one or more bronchopulmonary segments or extensive confluent bronchopneumonia. Cavities may be visible but are usually obscured by the extensive inflammatory process. Distant spreads of the process may occur early even though cavities are not visualized on the conventional chest film.

In the absence of effective treatment, tuberculous pneumonia advances relentlessly. There is progressive liquefaction of caseous material, with the formation of large cavities from which tubercle bacilli spread to adjacent and distant portions of the lungs. In the Negro, the course of the disease is usually rapid; most patients die within one year. A few Negroes develop chronic active tuberculosis, but this course is more common in white patients.

Before the use of streptomycin, the mortality from tuberculous pneumonia was very high both in white and nonwhite persons. In 1944, Shields (4) was unable to influence the mortality in the Negro by the use of pneumothorax. Other forms of collapse therapy did not appear to be successful when applied alone, although Schwartz and Moyer (6) stated that pneumoperitoneum was of value in tuberculous pneumonia before streptomycin became available. The experience of different authors is at variance on this point. Kirby, Simpson, and Creger (5) reported that pneumoperitoneum failed to control the disease in most of the 15

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[2] Presented in part at the annual meeting of the Southern Tuberculosis Conference in Miami Beach, Florida, September 11 and 12, 1952.

Negroes they treated with streptomycin. Schwartz and Moyer, however, found that pneumoperitoneum definitely improved the results when instituted six to eight weeks after treatment with streptomycin was begun.

Streptomycin alone has proved to be inadequate for the control of acute tuberculous pneumonia. The emergence to predominance of bacilli resistant to the drug results in clinical relapse after two or three months and the relapse usually leads to a fatal outcome. The introduction of para-aminosalicylic acid (PAS) and isoniazid has made it possible to maintain effective long-term antimicrobial therapy for the disease.

The present paper represents an account of the experience gained in treating 23 Negroes with acute tuberculous pneumonia. The aims of therapy were to prevent progression and to produce a chronic stable pattern of the disease. At an optimal time, attempts were made to close cavities or to prepare the patient for surgical treatment. Since the cases reported were admitted in the period from January, 1948, to January, 1951, various treatment regimens were employed. The role of the various drugs and procedures used will be discussed in an effort to outline a rational program for the management of acute tuberculous pneumonia.

OBSERVATIONS

Analysis of Material

Most of the 23 Negro patients in the study were in the age groups of eleven to thirty and forty-one to fifty. Seven of the 12 patients in the younger group were females and 5 were males. Only 2 of the 8 patients in the forty-one to fifty group were females. Of the 3 remaining patients, 2 were almost sixty years of age, and the third patient was thirty-eight. This distribution corresponds with the age groups associated with the highest mortality from tuberculosis.

Nineteen of the 23 patients were ill for three months or less when admitted to the hospital. One patient had been sick for five months before he was hospitalized; the remaining 3 patients suffered pneumonic spreads of disease from demonstrated cavities and waited longer than four months before hospitalization.

Symptoms have not been listed for members of this series for fever, cough, expectoration, and weight loss were present in all cases. Sputum positive for tubercle bacilli was obtained in every case. One patient did not develop a productive cough until he had been in the hospital for two weeks; tubercle bacilli were then found for the first time. Chest pain, hemoptysis, and night sweats were not prominent symptoms.

The aim in the management of these patients was to enable them to reach a chronic stable stage of their disease. In order to obtain this goal, bed rest, chemotherapy, and other measures which will be discussed were used. Our experience with the various therapeutic regimens used in an effort to cure these patients with exudative tuberculous lesions is summarized in figure 1.

Therapy Employed During the Acute Stage

Streptomycin without PAS: Six of the patients treated early in the series did not receive PAS together with streptomycin since PAS was not then available.

Most of these patients did badly; only 2 patients survived and but one improved significantly. The amount of streptomycin given to this group of patients varied; the average patient received 30 to 42 one-gram doses of streptomycin; one patient received only 10 gm. of streptomycin before his early death.

Streptomycin with PAS: The best results in the study were obtained in the patients treated with streptomycin and PAS; the dosage and duration of treatment with these drugs approached current standards. The patients in this group numbered 17; 11 of these patients also received pneumoperitoneum therapy. Only 2 of the 17 patients in this group are dead; however, 3 of the living patients are in the unimproved category. An analysis of figure 1 shows that the group which did not receive pneumoperitoneum therapy did better than the group receiving that treatment. It should be pointed out, however, that the patients who received pneumoperitoneum therapy, with 2 exceptions, had bilateral disease and were usually more severely ill than the others. The small number of cases in

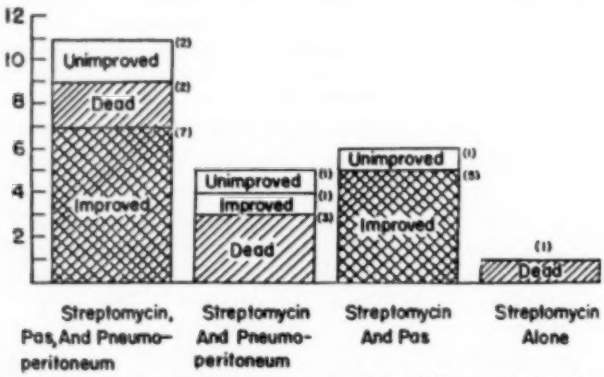

FIG. 1. Results of various treatment regimens. It should be noted that the best results were obtained when pneumoperitoneum was combined with streptomycin and PAS.

both groups also makes it difficult to draw any definite conclusions about the effectiveness of pneumoperitoneum therapy.

Pneumoperitoneum: The pneumoperitoneum was considered adequate (1) in 12 of the 16 patients in whom the procedure was employed; only one of these patients with satisfactory collapse is dead. Five of these patients now have sputum negative for tubercle bacilli, and 7 are considered to be good surgical prospects. Eleven of the patients have received streptomycin and PAS in addition to pneumoperitoneum.

Other procedures used during the acute phase: Artificial pneumothorax therapy was utilized in 5 early cases. Three of the patients in this group developed prompt spreads of disease, and the procedure had to be abandoned. The other 2 patients died of progressive disease.

Phrenic nerve crush was used as an adjunct in 3 of the pneumoperitoneum patients; this operation was employed when it was decided to maintain the pneumoperitoneum as the chief form of collapse therapy for a long period of time.

Measures Employed During the Chronic Phase

Once a patient has reached a chronic stable phase of the disease, he can then be managed as an ordinary case of chronic pulmonary tuberculosis. Surgical measures reserved for this stage of stability should not be employed prematurely because of the danger of spread of the disease. This is illustrated by the early death of one of the patients in the series who had a thoracoplasty three months after the onset of his disease when resolution of the acute pneumonic lesion was still incomplete.

Three of the patients in the series have had thoracoplasty. One patient died during the immediate postoperative period; another, mentioned above, was operated upon prematurely and subsequently died. One of these 3 patients is alive and well and his disease can be classified as inactive.

Two pulmonary resections have been performed on members of this series. One patient had an upper lobe lobectomy, with an excellent result; he was

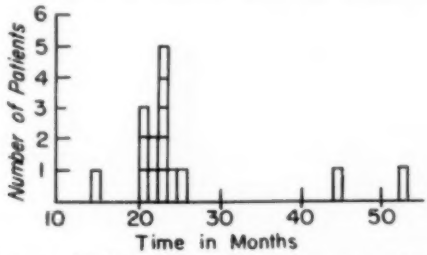

FIG. 2. Follow-up chart of living patients. Chemotherapy combined with pneumoperitoneum has enabled patients with tuberculous pneumonia to live longer than was previously possible.

operated upon more than one year ago and his disease is classified as *inactive*. The other patient developed cardiac arrest during pneumonectomy and died.

Thus, 2 of the patients whose disease had reacted favorably to treatment died as a result of major surgical procedures and are not listed among the 15 living patients in this study (figure 2)

Follow-up of Living Patients

Fifteen patients are still alive after periods ranging from fourteen to fifty months from the development of symptoms. All of the patients developed a stable pattern of disease during the course of their treatment. In 3 patients the disease is now classified as inactive; 2 had successful surgical treatment; and the third has received pneumoperitoneum. In 3 other patients, the disease is no longer under control, and the patients have been followed for periods ranging from fourteen to twenty-two months. The remaining 9 patients continue with chronic —apparently stable— tuberculosis, which in 8 of the 9 will probably be suitable for surgery in the future (figure 3).

Deaths

Eight of the 23 patients died of progressive tuberculosis: 4 patients died less than one year from onset of the disease; 3 patients experienced relapse after ap-

parent arrest of the lesions following eighteen to twenty-four months of treatment; and in the eighth patient relapse occurred after pneumonectomy.

Three of the 4 patients who died of acute spreading tuberculosis had severe bilateral involvement when treatment was first started. Since they were among the earliest members of the series, only one patient was given PAS and this was started one and one-half months after streptomycin was begun. In 2 cases pneumothorax was induced but was ineffective; important pleural complications developed in each instance. Thoracoplasty performed three months after admission did not arrest the spread of the disease in one patient.

Only one of the 3 patients who died of spreading disease after an initial period of stability had bilateral pulmonary involvement when she was first seen. The other 2 refused to accept surgical treatment at what appeared to be an optimal time. Para-aminosalicylic acid was not available when one of the patients was treated but the others received it in combination with streptomycin. Collapse therapy was used in all of the cases: 2 had pneumoperitoneum and phrenic nerve

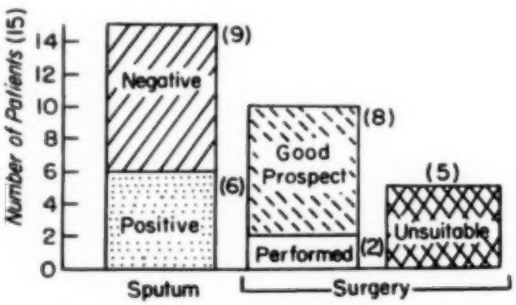

FIG. 3. Follow up chart of living patients. The majority of patients with tuberculous pneumonia have not only had their lives prolonged but also have been offered the prospect of permanent inactivation of their disease through surgery.

crush; the third had pneumothorax, which was unsatisfactory because of pleural complications.

DISCUSSION

The results summarized in this paper and elsewhere in the literature indicate that there have been great advances in the treatment of acute tuberculous pneumonia during the past few years. Although this mortality rate is much lower than that of the pre-chemotherapeutic era, it is still high when compared to results attained today with the drugs and procedures now available. Therapeutic progress is clearly demonstrated when one considers the changed prognosis for affected Negroes; only 4 of the patients in the present series died of spreading tuberculosis within one year of onset.

All of the patients admitted to the Tulane Tuberculosis Service at Charity Hospital with massive tuberculous pneumonia during 1952 were compared with the series reported in this paper. The new series was composed of 15 Negro and 4 white patients. There has been no fatality in the second group and all are classi-

fied as improved at present (April, 1953). These patients have received streptomycin, PAS, and isoniazid in combinations of two or three drugs on a long-term basis. Pneumoperitoneum therapy has been employed in many of these cases.

It is reasonable to expect that the results in the treatment of massive tuberculous consolidations will continue to improve. When the clinical course and management of the reported series of cases were reviewed, the factors which adversely influenced the outcome for some of these patients were singled out so that they might be corrected in the future.

Delay in the institution of therapy, whether due to the insensitiveness of the patient or lack of room in the hospital, led to excessive caseation and cavitation with spread of the disease both to nearby and distant parts of the lungs. Prompt therapy is essential for control of the pneumonia. Therapy, effective orally, is now available for the prehospitalization period. The aim of early treatment is to check pulmonary destruction and to prevent spread of disease. If these goals are attained, a chronic focal lesion will result and the therapeutic problem will be similar to that in other forms of chronic pulmonary tuberculosis except that a patient with antecedent tuberculous pneumonia probably has an inherently low natural resistance to the disease.

The treatment of patients with tuberculous pneumonia has been unsuccessful in the past. Bed rest and supportive measures are required, as they are for all patients with unstable tuberculosis, but these are inadequate for any decisive effect upon the pneumonic process. An agent or agents which will prevent the tubercle bacillus from multiplying is necessary. Streptomycin alone is not satisfactory, for the bacilli usually develop resistance before exudative phenomena have resolved. Consistently good results with drug therapy were first obtained when streptomycin and PAS were used concomitantly for long periods of time (6). The effectiveness of isoniazid makes it possible to maintain combined drug therapy even when the patient is intolerant to either streptomycin or PAS. In selected cases, it may be advisable to use all three drugs in an attempt to obtain a prompt and lasting effect upon the disease.

Although pneumoperitoneum was used in many cases of the present series, it is difficult to assess its value because other forms of therapy were used concomitantly. The results indicate that it was most effective in patients who also received streptomycin and PAS; the results were poor when it was given to patients who received streptomycin alone. Patients treated with streptomycin and PAS did as well as patients who received pneumoperitoneum in addition to both drugs. The results obtained in the present small series suggest that pneumoperitoneum is of secondary importance in treating acute tuberculous consolidations. An effective combination of antituberculous drugs is essential if good results are to be obtained. Improvement can usually be obtained without the use of collapse therapy. Other studies (5, 6) have suggested that pneumoperitoneum is of value when used with streptomycin alone. Since the duration of effectiveness of streptomycin was limited in most of the cases treated by Schwartz and Moyer (6), pneumoperitoneum must have been an important factor in obtaining their results.

The optimal time for the induction of pneumoperitoneum in patients with

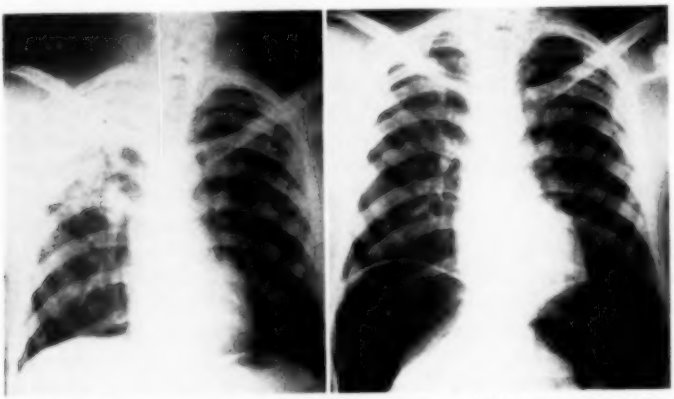

FIG. 4. (Left) Chest roentgenogram of E. B., a 20-year-old Negro male, taken October 19, 1950, reveals massive tuberculous pneumonia involving the right upper lobe. This patient was acutely ill when admitted and had been ailing for six weeks. He received streptomycin (a total of 84 gm.), para-aminosalicylic acid, and pneumoperitoneum.

(Right) Chest roentgenogram, May 12, 1952, reveals marked clearing of the pneumonic lesion. Sputum has been persistently negative for tubercle bacilli on concentrates and cultures for more than a year. Pneumoperitoneum is being continued. It is believed that surgery will not be needed.

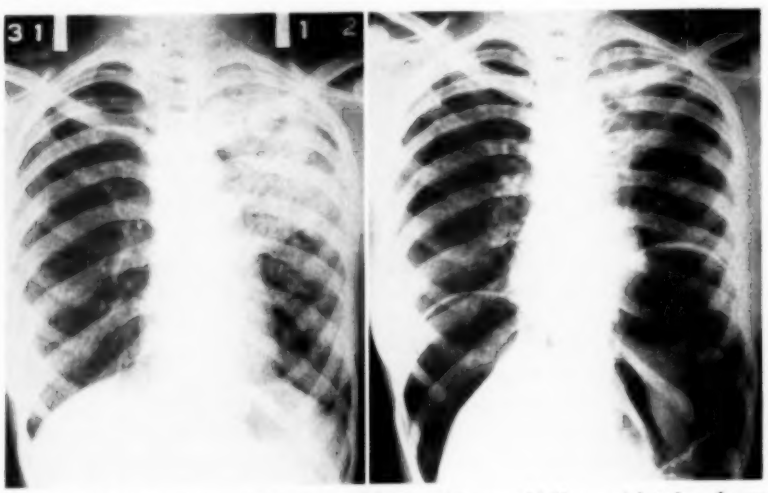

FIG. 5. (Left) Chest roentgenogram of M. F., a 16-year-old Negro girl, taken January 28, 1950, reveals tuberculous pneumonia involving the left upper lobe. This patient was acutely ill, temperature 104°F., when admitted; she had been ill for two months before admission. She received 42 gm. of streptomycin and para-aminosalicylic acid. Pneumoperitoneum was started in March, 1950, and a left phrenicectomy was performed a few weeks later.

(Right) Chest roentgenogram, May 23, 1952, shows considerable clearing of the pneumonic lesion. This patient's sputum is persistently negative for tubercle bacilli on concentrates and cultures but it is believed that surgery will still be necessary.

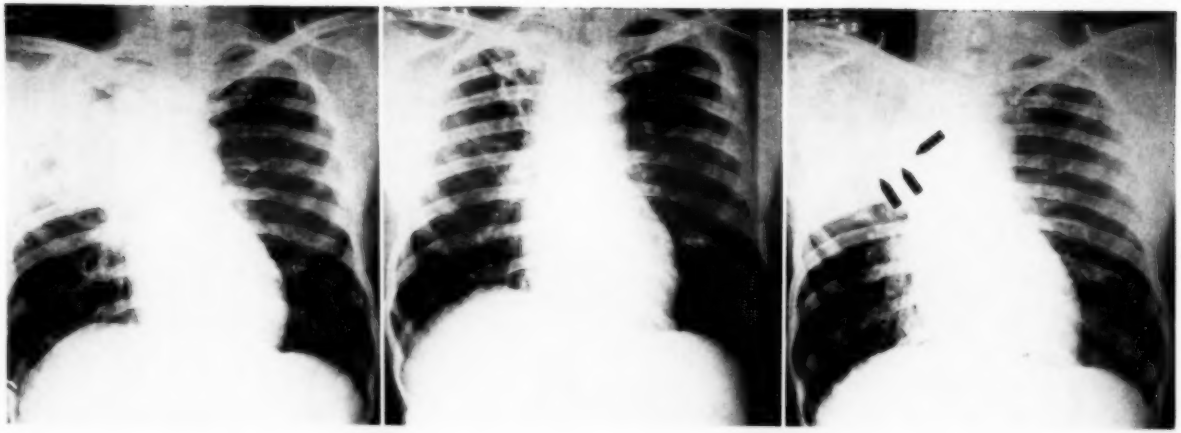

FIG. 6. (Left) Chest roentgenogram of J. S., a 46-year-old Negro male, taken September 23, 1950, reveals a tuberculous pneumonia involving the upper lobe of the right lung. This patient had been ill for six weeks and had a temperature of 99.9°F. when admitted. He received 42 gm. of streptomycin with para-aminosalicylic acid. Pneumoperitoneum therapy was not given.

(Center) Chest roentgenogram, May 16, 1951, shows considerable clearing of previously described lesion with contraction of the right upper lobe; however, excavation is now visible in the right first interspace. A right Paulino type of thoracoplasty (three- and one-half-rib thoracoplasty with suture constriction of the upper lobe to obliterate the cavity area) was performed a few days afterwards.

(Right) Chest roentgenogram, March 24, 1952, was taken ten months after the Paulino thoracoplasty. The patient's sputum is persistently negative for tubercle bacilli on concentrate and culture. He has been discharged, and his lesion is classified as "inactive."

acute tuberculous pneumonia is not clear. It appears useless to employ pneumoperitoneum very early, when the lesion is fully consolidated. It has been suggested that six to eight weeks after the institution of drug therapy is the proper time for induction if lobar contraction, which frequently complicates pneumoperitoneum, is to be avoided. It is unlikely that any set time will prove satisfactory in all cases. The suggestion is made that pneumoperitoneum be delayed until there has been considerable resolution of the consolidation and a stable pattern of pulmonary infiltration and destruction has become apparent. This state is more likely to occur three to six months after institution of drug therapy than after the briefer periods just mentioned.

Other collapse measures such as artificial pneumothorax and thoracoplasty have been used with poor results in the early therapy of large acute tuberculous consolidations. Pneumothorax often leads to serious pleural complications which increase the severity of the illness. Thoracoplasty at this stage adds new hazards of operative risks and impaired ventilatory capacity and does not affect excavation in the underlying pneumonic lesion.

Surgery can only be successfully employed when the acute tuberculous pneumonia has been converted into chronic tuberculosis. It is no longer necessary to perform early pulmonary resection for any apparently unilateral lesion as in 2 cases reported by Kirby, Simpson, and Creger (5). Long-term chemotherapy will almost always bring the patient's disease to a chronic stage during which surgery can be safely carried out if it is indicated; less extensive procedures such as lobectomy and Paulino thoracoplasty (2, 3) will then be possible since marked resolution usually occurs.

Thus, long-term antimicrobial therapy and the judicious induction of pneumoperitoneum enable patients with acute massive tuberculous pneumonia to reach a chronic phase of their disease. In certain rare instances, the patient will die of acute spreading tuberculosis in spite of all therapeutic efforts. Fortunately such cases are uncommon. The majority of patients reach a stage during which their treatment problems are the same as for other patients with chronic pulmonary tuberculosis. The patients with focal unilateral disease can be treated with pulmonary resection or thoracoplasty if surgery is indicated. An occasional case will become inactive on drug therapy and pneumoperitoneum. Patients with extensive spreads of disease to the opposite lung present the most difficult problem of all; the stability of the opposite lung is never assured and cavities are often present. Such patients are usually given long-term drug therapy and pneumoperitoneum. These measures enable the patient to pass through the phase of acute tuberculous consolidation, but the disease will often escape from control after a long period and will eventually prove fatal. This has happened in 3 patients in the present series and is occurring in 3 others. Reliance upon drugs and pneumoperitoneum for indefinite periods will often lead to disappointment because of the severity of the lesions and the poor resistance of these patients. The best prospect for most of these patients is that their disease will ultimately become suitable for surgical treatment.

SUMMARY

Streptomycin and para-aminosalicylic acid (PAS) should be used to treat tuberculous pneumonia for a long period of time in order to enable the patient to reach a state of stability. Isoniazid may be advantageously combined with PAS and streptomycin in certain cases or it may be used in the event of PAS or streptomycin toxicity.

No form of surgical treatment is indicated for acute tuberculous pneumonia until the lesion has been stable for several months. The place of pneumoperitoneum therapy in the treatment of acute tuberculous pneumonia remains undetermined.

Patients with acute tuberculous pneumonia should be treated early and energetically. The great majority of such patients can be successfully managed through the acute pneumonic period. When exudative phenomena have been controlled, the patient is left with chronic active tuberculosis which must be treated by appropriate measures.

The prognosis of acute tuberculous pneumonia has improved greatly as a result of the combined use of streptomycin and PAS. Many patients are now able to reach a stage during which corrective surgery can be performed. This is in sharp contrast to the fate of these patients in the pre-streptomycin era.

SUMARIO

La Neumonía Tuberculosa Aguda en la Raza Negra

A fin de capacitar al enfermo para que alcance una fase de estabilidad, hay que usar durante un período prolongado de tiempo la estreptomicina y el ácido para-aminosalicílico (PAS) para tratar la neumonía tuberculosa. En ciertos casos, cabe combinar provechosamente la isoniacida con el PAS y la estreptomicina o puede usarse para reemplazar una de dichas drogas si ésta produce reacción tóxica.

No está indicada ninguna forma de tratamiento quirúrgico antes de que la lesión haya permanecido estabilizada por varios meses. Está aun por determinar el puesto del neumoperitoneo terapéutico en el tratamiento de la neumonía tuberculosa aguda.

A los enfermos con este síndrome hay que atenderlos tempranamente y que tratarlos con toda energía. La inmensa mayoría de ellos pueden ser atendidos con éxito en el período neumónico agudo. Una vez dominados los fenómenos exudativos, el paciente queda con una tuberculosis activa crónica que tiene que ser tratada apropiadamente.

El pronóstico de la neumonía tuberculosa aguda ha mejorado considerablemente a consecuencia del empleo combinado de la estreptomicina y del PAS. Muchos enfermos ya pueden hoy día llegar a una etapa en la que cabe ejecutar cirugía reparadora, lo cual es muy distinto del destino que les correspondía en la era pre-estreptomicínica.

RESUME

Pneumonie tuberculeuse aiguë chez le Nègre

Dans le traitement de la pneumonie tuberculeuse, l'administration de la streptomycine et l'acide para-aminosalicylique (PAS) doit être poursuivie pendant très longtemps pour permettre au malade d'atteindre le stade de stabilisation. L'isoniazide peut être avantageusement associée au PAS et à la streptomycine, dans certains cas, ou encore elle peut être utilisée en remplacement d'une de ces drogues dans le cas où celle-ci produirait une réaction toxique.

Aucune forme de traitement chirurgical n'est préconisée jusqu'au moment où la lésion

est stabilisée pendant plusieurs mois. Le rang occupé par le pneumopéritoine dans la thérapie de la pneumonie tuberculeuse aiguë reste indéterminé.

Les malades présentant ce syndrome doivent être traités précocement et énergiquement. La plupart de ces malades peuvent, être traités avec succès pendant la phase aiguë de la pneumonie. Quand les phénomènes exsudatifs ont été jugulés, le malade conserve une tuberculose chronique en activité qui doit être traitée par des mesures appropriées.

Le pronostic de la pneumonie tuberculeuse aiguë s'est amélioré considérablement à la suite de l'emploi de l'association streptomycine-PAS. De nombreux malades sont désormais capables d'atteindre un stade au cours duquel une intervention chirurgicale corrective peut être pratiquée. Ces faits présentent un contraste marqué avec le sort des malades de l'ère "pré-streptomycine."

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A STUDY OF IRREGULAR DISCHARGES IN A VETERANS ADMINISTRATION TUBERCULOSIS HOSPITAL[1]

JOSEPH F. TEDESCO

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INTRODUCTION

The records of 100 consecutive patients who received irregular discharges at the Veterans Administration Hospital, Castle Point, New York, between February 18, 1951, and June 29, 1951, have been reviewed. During this period, only 38 patients (27 per cent of the total discharges) received regular discharges. (This is exclusive of transfers to other hospitals and deaths.)

The problem of irregular discharges has been causing considerable concern among all persons involved in the care of tuberculous patients. A brief review of some previous studies of irregular discharges, including the reasons which patients gave for leaving irregularly, would be of interest before analyzing the discharges at Castle Point.

A comprehensive study was done by William B. Tollen (1), who found that the rate of irregular discharges from non-Veterans Administration hospitals was comparable to that of the Veterans Administration hospitals. The percentage of irregular discharges as compared with regular discharges was slightly smaller in the former, but transfers to other hospitals were included among the regular discharges in the statistics of non-Veterans Administration hospitals.

Between 1942 and 1947, 52 to 72 per cent of the discharges of tuberculous patients from all Veterans Administration Hospitals were irregular in nature.

A study was done by the Veterans Administration in 1943 (2) in which the patients gave the following reasons for irregular discharges: family business or financial problems, 39 per cent; dissatisfaction with hospital rules and procedures, 18 per cent; inability to find absorbing activities in the hospital, 11 per cent; ability to receive adequate care at home, 9 per cent; objection to reduced pension, 7 per cent; failure to return from leave, 5 per cent; dissatisfaction with medical treatment, 3 per cent; fear of action by hospital board of discipline, 3 per cent; alcoholism, 3 per cent; and miscellaneous, 2 per cent.

All irregular discharges of tuberculous patients from Veterans Administration hospitals in July, 1947, were reviewed. In that month, 569 patients were discharged irregularly. In the same month there were 658 regular tuberculous discharges. Forty-five per cent of the patients discharged irregularly in July, 1947, had had previous irregular discharges; 33 per cent were hospitalized less than one month; 75 per cent left within six months after admission.

Between October, 1947, and January, 1948, 533 of the patients who received irregular discharges were interviewed by social workers. Questionnaires were completed by 401 of these ex-patients. The factors leading to irregular discharges were divided into three main divisions: (1) those originating outside the hospital and related to personal, social, and economic status; (2)

[1] From the Veterans Administration Hospital, Castle Point, New York.

those originating within the hospital; (3) those emanating within the personality of the veteran. According to the social workers, the irregular discharges were divided equally among these three groups, but 80 per cent of the patients attributed their discharges to factors arising within the hospital.

Studies by other investigators revealed that the same factors were operative in their patients. Galinsky and Brownstone (3) blame "(*a*) inability to adjust to sanatorium routine, (*b*) sociological factors, e.g., disruption of the home and financial matters, (*c*) inability to continue further treatment." Johnson (4) states that, "80 per cent of irregular discharges had emotional or behavior problems." Frankel (5) gives the reasons, "(*a*) concern over affairs at home, and (*b*) problems of hospital adjustment." Banyai and Cadden (6) list various reasons which include social, economic, and personal factors. Bobrowitz (7) studied 70 patients and states that, "42 per cent left because of personal problems or emotional factors, 25 per cent felt that further hospital treatment was unnecessary, 17 per cent expressed specific dissatisfaction with the sanatorium, 11 per cent were dissatisfied with medical treatment, the remaining 5 per cent gave miscellaneous reasons."

Tollen suggested that the problem should be attacked by the combined forces at the hospital. His recommendations include utilization of medical social service during the period prior to hospital admission as well as during hospitalization; patient orientation after admission; strengthening of the patient-doctor relationship; availability of psychiatric service; coordination of medical care with vocational training; rehabilitation and physical medicine; and increased use of special services.

OBSERVATIONS

Report on Castle Point Cases

Before analyzing the series of 100 irregular discharges at Castle Point Hospital, it should be noted that many of the suggestions listed by Tollen have been in effect for several years at this hospital. All newly admitted patients are given an orientation course which includes lectures by the assistant chief of the tuberculosis service, the clinical psychologist, and by members of the following departments: social service, rehabilitation, nursing, dietetic, and special services. Wherever possible all newly admitted patients are interviewed individually by a member of the social service department and by the clinical psychologist. Since the usual patient load at this hospital is approximately 580, two social service workers cannot see all of the newly admitted patients. For the last two years all newly admitted patients who have had any personal problem which might lead to an irregular discharge were immediately referred to both the clinical psychologist and the social service department. Patients who had a history of previous irregular discharges were also given the benefit of early consultations with members of these departments. The ward physicians also discuss problems and treatment as well as personal problems with the patient when necessary.

In the Castle Point series, the preponderance of patients in the younger age group is striking. Only 17 patients were veterans of World War I. Eighty-one patients were veterans of World War II, and 2 patients had peacetime service

prior to World War II. At the time this report was made, 69 per cent of the patients in the hospital were either veterans of World War II or of peacetime service. The percentage of irregular discharges in the younger patient is higher than would be expected from the number hospitalized in that age group.

It is interesting to note that the illnesses of 75 per cent of the patients were designated as "service-connected," i.e., attributable to their military service. Of these, 73 were in the World War II group. These patients are aware of the fact that they must be readmitted after ninety days if they desire treatment. Fourteen of these patients were readmitted within a six-month period after June, 1951; 13 of these readmissions were "service-connected." The one "non-service-connected" patient was readmitted as an emergency. Fifty-six of these patients have had previous irregular discharges. Some of them have had as many as seven such discharges. These irregular discharges have not only been from Veterans Administration hospitals but also from private, city, or state hospitals (table 1).

TABLE 1

IRREGULAR DISCHARGES FROM VETERANS ADMINISTRATION HOSPITALS AND FROM PRIVATE, CITY, AND STATE HOSPITALS

NUMBER OF PREVIOUS IRREGULAR DISCHARGES	NUMBER OF PATIENTS	CASTLE POINT ALONE	OTHER VA HOSPITALS	CITY, STATE, OR PRIVATE HOSPITALS	VA AND OTHER INSTITUTIONS	MORE THAN ONE VA HOSPITAL
1	28	6	11	11	—	—
2	17	2	2	3	6	4
3	5	—	—	1	3	1
4	2	—	—	—	2	—
5	1	—	—	—	1	—
6	1	—	—	—	1	—
7	2	—	—	—	—	2
Total	56	8	13	14	13	7

The preponderance of previous discharges in "service-connected" patients may be seen in table 1. Forty-eight (64 per cent) of the patients who have had previous irregular discharges were in the "service-connected" group.

A review of the patients' clinical records reveals the following reasons for irregular discharges:

(*a*) Fifteen left after an alcoholic episode; (*b*) 14 patients were restless and told either their ward physician or the clinical psychologist that "they never could adjust to treatment"; (*c*) 13 patients failed to return from a furlough; (*d*) 7 left after they could not obtain furlough; (*e*) 6 patients left because they "felt that they could do just as well at home"; (*f*) 5 left because they desired hospitalization closer to home; (*g*) 4 left while the decision of a hospital board of discipline following infraction of rules was pending; (*h*) 3 patients left because they had "only expected a brief period of observation" to determine activity of pulmonary disease; (*i*) 4 left after they were not granted discharges as having achieved maximum hospital benefits; (*j*) 2 patients left because of each

of the following reasons: family difficulties at home, generally dissatisfied with hospital routine, entirely indifferent to the need for hospitalization, desire to go home after transfer from a military hospital; (*k*) one patient was discharged by hospital board of discipline; (*l*) in 11 cases records failed to show any reason for leaving without permission of the staff. (These patients were apparently satisfied with hospital treatment until they suddenly decided to leave); (*m*) 11 patients left for miscellaneous reasons, e.g., 2 left after being moved from "their" bedroom, one left because he would not accept a seven-day furlough after he had requested eight days, one left after having had to appear four times before boards of discipline, another left because he felt that the "World War II" patients were rejecting him, one patient was admitted for a non-tuberculous condition and left after seven days. This case cannot be called a failure in the treatment for tuberculosis.

The small percentage of patients who expressed dissatisfaction with medical treatment before leaving is striking. Some of the group of 11 who left without expressing the reason might have been dissatisfied with their medical treatment.

It is interesting to note that the majority of irregular discharges occurred within the first five months of hospitalization. Fifty-seven per cent of the patients left within the first six months after hospitalization. Eleven of these left in the first month, 12 in the second month, 8 in the third month, 12 in the fourth month, 10 in the fifth month, and 4 in the sixth month after admission to the hospital. Eighty-three per cent of the patients who were discharged irregularly left within the first year. Of the 57 who left in the first six months, 34 had records of previous irregular discharges.

Social service records were reviewed and the reasons why patients received irregular discharges were available in 71 patients. Twenty-nine patients were not seen by the social service department during the hospitalization period reported. The reasons for leaving were divided into three groups;

(*a*) Twenty-two patients left because of difficulties which arose in the patient's home (financial problems; desired treatment closer to home); (*b*) 29 discharges were attributed to difficulties resulting from the patient's personality (restlessness, impression that further bed rest was not needed, lack of insight into the seriousness of their disease, psychopathic personality); (*c*) 20 patients left because of inability to follow hospital regulations. Thirteen of these 20 patients left before a board of discipline could be convened following breach of hospital rules; 3 others left because they objected to certain hospital regulations; one patient was discharged by the disciplinary board; one patient left after he was not discharged as having achieved maximum hospital benefits; 2 others left after only a few days of hospitalization, stating that they had expected only a brief period of observation.

These figures parallel those reported by other observers, namely, that, in the overwhelming majority of irregular discharge cases, the departure from the hospital can be attributed to either factors arising within the patient or in the patient's home situation. Only a very small percentage can be directly attributed to factors originating in the hospital.

COMMENT

The problem of irregular discharges has been a matter of deep concern to those involved in the treatment of tuberculous patients. This problem is not peculiar to the Veterans Administration alone because statistics show comparable rates of irregular discharges from non-Veterans Administration hospitals. It is also evident that only a small percentage of the irregular discharges reported by both types of hospitals can be attributed to dissatisfaction with hospital treatment. In the majority of patients, factors originating outside the hospital, for example, social and economic problems and factors originating within the personality of the patient, are responsible for the irregular departure from the hospital.

In spite of a concerted effort by various members of the different services at the Castle Point hospital to indoctrinate and properly orient all newly admitted patients, the rate of irregular discharge is still excessive. One important factor is that some patients are not properly prepared for a prolonged period of hospitalization prior to admission. The proper education of each person in whom the diagnosis of active tuberculosis is made should begin with the physician who first makes the diagnosis. For various reasons, some of which may be beyond the control of the diagnosing physician, the new patient does not obtain a proper insight into the various aspects of tuberculosis. Due to this fact certain patients never do become adjusted to the need for prolonged hospitalization. Several patients stated that they had been advised "that they would only be hospitalized for a brief period of observation" before they were sent to this hospital. Upon being informed that they would require prolonged hospitalization for treatment of active tuberculosis, these patients left against medical advice. Another attitude which is frequently encountered when new patients are interviewed shortly after transfer from another hospital is "I was told that I would be discharged in several weeks." On occasion it may be extremely difficult to impress this type of patient with the need for prolonged hospitalization.

More attention should also be given to taking care of various problems in the patient's home before he enters the hospital. Any financial and personal problems which might require the attention of the patient should be settled with the assistance of local social agencies. Patients are advised on admission to this hospital that they should not hesitate to discuss any personal matters with their ward physician or a member of the social service department. In spite of this advice, the fact remains that some patients are forced to accept irregular discharges because "urgent problems have suddenly arisen at home which cannot be handled by the social service department."

Another large group of patients who require special attention are those who state that "they can not stand long periods of hospitalization." These patients are all interviewed by the clinical psychologist as well as by the ward physician in an attempt to prevent the irregular discharge. In spite of these contacts, most of this group of patients cannot be persuaded to follow prolonged hospital treatment.

The third group of patients in whom the number of irregular discharges is high is the one in which the patients have difficulty in adhering to hospital regulations.

One recommendation which is frequently made to attempt to prevent irregular discharges is that the patients should be granted more frequent furloughs. Statistics from this hospital show that 13 patients failed to return from a furlough. Seven patients left because they could not obtain furloughs. Apparently, patients will still leave irregularly in spite of a liberal furlough policy.

Another hospital regulation which is frequently a cause of irregular discharge is the one forbidding the use of alcholic beverages. Fifteen patients left rather than face a hospital board of discipline after a drinking episode.

It is evident that the problem of irregular discharge will always be present because some patients who need prolonged treatment will be unable to complete the prescribed period of hospitalization because of the reasons given above. With more concerted effort by everyone who comes into contact with each patient, the number of irregular discharges can be reduced; however, it cannot be eliminated entirely.

SUMMARY

The cases of 100 patients who were consecutively discharged irregularly from a Veterans Administration hospital were reviewed.

The majority of irregular discharges reported by previous writers and in the present study can be attributed to factors arising outside of the hospital itself rather than from the treatment received within the tuberculosis hospital.

SUMARIO

Estudios de las Altas Irregulares en un Hospital para Veteranos

Repásanse aquí 100 altas irregulares consecutivas de un hospital para veteranos de los E. U. A.

Preséntase también un breve resumen de los estudios realizados por investigadores anteriores.

Las mayoría de las altas irregulares descritas por otros observadores y en el hospital actual pueden atribuirse a factores procedentes del exterior del hospital más bien que relacionados con el tratamiento recibido en éste.

RESUME

Une étude des réformes irrégulières dans un hôpital de "Veterans Administration"

Une série de cent cas consécutifs de réformes irrégulières dans un hôpital de "Veterans Administration" a été étudiée.

Un bref résumé des études effectuées par des investigateurs précédents est présenté.

La majorité des réformes irrégulières rapportées par des observateurs précédents ainsi que celles de cet hôpital peut être attribuée à des facteurs se manifestant hors de l'hôpital plutôt qu'au traitement reçu.

Acknowledgment

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A PHARMACOLOGIC TRIAL OF PARA-ETHYLSULFONYL BENZALDEHYDE THIOSEMICARBAZONE (BERCULON B) IN HUMANS[1,2]

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INTRODUCTION

Numerous reports have been made regarding the experimental and clinical aspects of the effectiveness of thiosemicarbazones in the treatment of tuberculosis. Of the series of thiosemicarbazones investigated by Domagk and his colleagues, para-acetamido benzaldehyde thiosemicarbazone (thiacetazone[3]) was considered to be the most effective. A critical review of the results obtained in Germany has been made by Hinshaw and McDermott (1) and the pharmacology of thiacetazone in humans has been well described by Simmons and his associates (2). Recently, however, experimental evidence has suggested that para-ethylsulfonyl benzaldehyde thiosemicarbazone (Berculon B[4]) is more effective than thiacetazone. Thus, Hamré and his associates (3) showed that, of 100 thiosemicarbazones and related compounds tested, only 8 showed significant reproducible activity, the effect of ethylsulfonyl substitution being superior to that obtained by substitution with the acetyl-amino group. The superiority of Berculon B in experimental tuberculous infection in mice has also been demonstrated by Hoggarth and Martin (4). Francis and his associates (5) have shown that there is wide variation in the dosage of Berculon B tolerated by various animal species; monkeys and mice were shown to tolerate 500 mg. per kg. twice daily, whereas in dogs, death from pulmonary edema occurred with a dose of 25 mg. per kg. Greenberg and Thorp (6) have observed in eighteen persons receiving Berculon B that the concentrations obtained in the blood were approximately three times as high as those following a similar dose of Berculon A (thiacetazone). This confirms results obtained in experimental animals.

It seemed reasonable before embarking on a clinical trial of Berculon B to endeavor to discover as accurately as possible the pharmacology of the drug. This report records the results of a trial set up under the auspices of the Research Committee of the Tuberculosis Society of Scotland to determine the pharmacology of Berculon B in humans. No attempt was made in this trial to evaluate the therapeutic activity of Berculon B.

METHODS

Forty patients (21 male and 19 female) suffering from tuberculosis were selected, 10 patients from each of four hospitals—Robroyston Hospital, Ruchill Hospital, Lochmaben

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[1] From Robroyston Hospital, Glasgow; Lochmaben Sanatorium, Dumfriesshire; Ruchill Hospital, Glasgow; Royal Victoria Hospital, Edinburgh; and the Department of Tuberculosis, University of Edinburgh.

[2] A report to the Research Committee of the Tuberculosis Society of Scotland.

[3] Known in the United States as amithiozone.

[4] "Berculon" is the registered trademark of Imperial Chemical (Pharmaceuticals) Ltd.

Sanatorium, and the Royal Victoria Hospital, Edinburgh. Patients were then allotted at random to four treatment groups as follows:

Group I: (9 patients) who received 1 mg. per kg. twice daily
Group II: (11 patients) who received 1.25 mg. per kg. twice daily
Group III: (11 patients) who received 1.5 mg. per kg. twice daily
Group IV: (9 patients) who received 2 mg. per kg. twice daily

It had already been ascertained (6) that Berculon B could be administered for a period of three months at a dosage of 1 mg. per kg. twice daily without significant toxicity. It was considered that examination of the toxicity and concentration of the drug in the blood over the range 1.0 to 2.0 mg. per kg. twice daily would yield significant evidence of the pattern of toxicity of the drug.

Half-dosage was given for seven days prior to the commencement of the trial and full dosage was continued for eighty-one days, or until such time as toxicity warranted cessation of administration of the drug.

It is considered possible that the initiation of full dosage after only seven days at half-dosage may have been responsible for the occurrence of toxic symptoms, chiefly vomiting, in the early weeks of treatment. Most authors recommend the gradual increase in dosage over several weeks. For instance, Ptaszynski and Salvi (7) did not administer full dosage of 200 mg. daily of thiacetazone until four weeks had elapsed.

The following determinations were made at weekly intervals on two occasions prior to commencement of treatment and at weekly intervals thereafter in the higher dosage groups (*III* and *IV*) and fortnightly in *Groups I* and *II*.

(*1*) Estimation of hemoglobin, erythrocyte and leukocyte count
(*2*) Blood urea
(*3*) Serum bilirubin
(*4*) Bromsulfalein clearance test
(*5*) Urine albumin and microscopic examination of sediment
(*6*) Body weight

Greenberg and Thorp (6) have observed that the maximum blood concentration of Berculon B after a single dose occurs approximately four hours later. Blood samples were taken for estimation of blood concentration of the drug immediately before the morning dose, i.e., twelve hours after the previous dose, and four hours later. In this way, the maximum and minimum blood concentrations were estimated in each person. The groups were sampled as follows:

Group I: Sampled on days 5, 12, 25, 53, and 81
Group II: Sampled on days 5, 10, 18, 29, 53, and 81
Group III: Sampled on days 4, 8, 14, 22, 32, 52, and 80
Group IV: Sampled on days 3, 7, 11, 17, 24, 33, 52, and 80

Estimations of the Berculon B concentrations in blood samples were made spectrophotometrically (8) by Mr. J. M. Thorp of the Research Department of Imperial Chemical Industries Ltd. (Dyestuffs Division). It had previously been ascertained that no significant loss of the drug occurred during the time which elapsed between the withdrawal of blood and the time of estimation of the sample (up to four days), provided that samples were stored in the dark.

OBSERVATIONS

Incidence and Nature of Toxic Symptoms

The incidence and nature of toxic symptoms and their relationship to dosage are shown in tables 1 and 2. It will be seen that it was necessary to stop the drug because of toxic symptoms in 18 of 40 patients (45 per cent). The most frequently observed major toxic symptoms were gastrointestinal disturbances and the occurrence of progressive anemia; malaise, depression, and lassitude were also frequently observed.

TABLE 1

CLINICAL DATA, DOSAGE, AND BLOOD CONCENTRATIONS OF THIOSEMICARBAZONE (BERCULON B)

CASE NUMBER	HOSPITAL	AGE	SEX	WAS DRUG STOPPED	DAYS OF TREATMENT	REASON FOR STOPPING DRUG	MEAN BLOOD CONCENTRATION OF BERCULON B	MEAN INCREASE IN 4 HRS. FOLLOWING DOSE	STAGE OF DISEASE	DOSAGE TWICE DAILY
							mg. per 100 ml.	*mg. per 100 ml.*		*mg. per kg.*
1	A	24	F	Yes	8	Vomiting	(0.18)	0.03	Intermediate	1.0
2	A	21	F	No	81	—	0.21	0.03	Advanced	
3	A	15	F	No	81	—	0.22	0.02	Intermediate	
11	C	28	F	No	81	—	0.10	0.06	Advanced	
12	C	45	F	No	81	—	0.11	0.07	Advanced	
13	C	47	F	No	81	—	0.21	0.13	Intermediate	
21	D	41	M	No	81	—	0.22	0.08	Advanced	
22	D	38	M	No	81	—	0.10	0.07	Advanced	
23	D	40	M	No	81	—	0.15	0.06	Advanced	
							0.17	0.06		
4	A	32	F	Yes	21	Vomiting	0.43	0.06	Intermediate	1.25
5	A	28	F	Yes	21	Vomiting	0.33	0.11	Early	
6	A	36	F	No	81	—	0.21	0.10	Advanced	
7	A	29	F	Yes	8	Vomiting	0.47	0.17	Advanced	
24	D	36	M	No	81	—	0.16	0.11	Advanced	
25	D	28	M	Yes	28	Malaise and depression	0.23	0.12	Advanced	
26	D	34	M	No	81	—	0.24	0.11	Advanced	
27	D	27	M	No	81	—	0.22	0.07	Advanced	
31	B	26	F	Yes	18	Hemolytic anemia	0.41	0.06	Advanced	
32	B	26	F	No	81	—	0.20	0.09	Advanced	
33	B	16	F	Yes	56	Hemolytic anemia	0.30	0.11	Early	
							0.29	0.10		

8	A	21	M	Yes	32	Vomiting	0.47	0.16	Intermediate	
9	A	21	M	Yes	32	Vomiting	0.55	0.11	Early	
10	A	18	M	No	81	—	0.49	0.11	Intermediate	
14	C	23	M	No	81	—	0.29	0.11	Advanced	
15	C	23	M	Yes	19	Progressive anemia	0.25	0.12	Advanced	
16	C	33	M	No	81	—	0.20	0.14	Advanced	1.50
17	C	37	M	No	81	—	0.36	0.11	Advanced	
34	B	32	M	Yes	24	Progressive anemia	0.45	0.16	Intermediate	
35	B	40	M	Yes	31	Impaired liver function	0.54	0.10	Intermediate	
36	B	27	F	Yes	52	Progressive anemia	0.35	0.12	Advanced	
37	B	38	M	No	81	—	0.34	0.09	Intermediate	
							0.39	0.12		
18	C	32	F	Yes	52	Lassitude and depression	0.28	0.16	Advanced	
19	C	22	F	No	81	—	0.34	0.18	Advanced	
20	C	29	F	Yes	25	Vomiting	0.42	—	Advanced	
28	D	27	M	No	81	—	0.39	0.21	Advanced	
29	D	53	M	Yes	46	Hemolytic anemia	0.30	0.12	Advanced	
30	D	29	M	No	81	—	0.48	0.08	Advanced	2.0
38	B	37	F	Yes	30	Hemoptysis with prolonged clotting time	0.56	0.20	Intermediate	
39	B	49	M	No	81	—	0.61	0.18	Advanced	
40	B	24	F	Yes	45	Nausea and vomiting	0.71	0.10	Advanced	
							0.45	0.15		

Gastrointestinal symptoms: Gastrointestinal symptoms were observed at some time during treatment in 21 patients (52.5 per cent). The symptoms were sufficiently severe in 9 patients (22.5 per cent) for the drug to be stopped. Vomiting of a severe type was the leading symptom in all of the 9 cases. Such symptoms occurred as early as the first week in 6 instances and as late as the tenth week in one case. Of the 9 cases in which the drug had to be stopped, dosage had to be withdrawn in 7 instances before the end of the third week.

Hemopoietic system: A fall in *hemoglobin* and *erythrocytes* was recorded in 34 cases. A fall in hemoglobin exceeding 2 gm. was observed in 21 cases (52.5 per cent). The fall was usually observed first in the third and fourth weeks and was maximal at the eighth or ninth week. The average maximum fall recorded was 3.0 gm. (0.6 to 6.0 gm.) Fall in hemoglobin and erythrocyte count was accompanied in 18 cases by an increased concentration of serum bilirubin and by an

TABLE 2

NATURE AND INCIDENCE OF TOXICITY OF THIOSEMICARBAZONE (BERCULON B)

TOXIC SYMPTOMS	1.0 MG. PER KG.	1.25 MG. PER KG.	1.5 MG. PER KG.	2.0 MG. PER KG.	TOTAL
Gastrointestinal	2 (1)	6 (4)	7 (2)	6 (2)	21 (9)
Progressive anemia	2	3 (2)	9 (3)	7 (1)	21 (6)
Exanthemata	—	2	3	1	6
Liver damage (bromsulfalein test)	—	—	3 (1)	5	8 (1)
Conjunctivitis	1	2	4	3	10
Malaise and depression	3	4	5	7 (1)	19 (1)
Lassitude	4	4	3	4	15
Thirst	1	—	—	—	1
Tightness of breasts	1	—	—	2	3
Hemoptysis with prolonged clotting time	—	—	—	1 (1)	1 (1)

Figures in parentheses show number of patients in whom toxicity was sufficiently severe to cause stoppage of drug.

excess of urobilinogen in the urine in 10 cases. In most cases the hemoglobin concentration increased after the ninth or tenth week despite continuation of treatment.

Progressive anemia was sufficiently severe in 6 cases to necessitate cessation of treatment and, in 3 of these cases, an acute hemolytic episode occurred. Transfusion was required in one of these cases.

The toxic effect upon the hemoglobin was very slight in a dose of 1.0 mg. per kg. twice daily and also in those patients who received 2.0 mg. per kg. twice daily. This apparently anomalous finding may be explained by the fact that 8 of the 9 patients in the latter group had a low initial hemoglobin.

A slight fall in *leukocytes* was observed in 25 cases. No cases of agranulocytosis occurred.

In one patient who received 2.0 mg. per kg. twice daily, a severe hemoptysis occurred and the *blood clotting time* was found to be sixteen minutes. After this episode the blood clotting time was tested in 24 patients and in 6 was found to be

prolonged. The prothrombin time was estimated in 9 more patients and no abnormality was found.

Liver dysfunction: There are widely different opinions regarding the degree of liver dysfunction which occurs during thiosemicarbazone therapy and many investigators have employed numerous liver function tests simultaneously in order to determine the occurrence of liver dysfunction. In this connection, it is of interest to note that Ptaszynski and Salvi (7), employing 200 mg. daily of thiacetazone for an average of 8.5 weeks and carrying out serum alkaline phosphatase, thymol turbidity, and thymol flocculation tests, serum colloidal gold, and icteric index in twenty-four men found no evidence of liver damage.

In the present trial, liver function was estimated by the bromsulfalein clearance test. Falk (9), who has studied this question closely, states that this test gives a good measure of the liver damage caused by thiacetazone. Interference with clearance of bromsulfalein was observed in 8 cases, in 3 instances with dosage of 1.5 mg. per kg. twice daily and in 5 instances in the 2.0 mg. per kg. group.

The functional impairment was sufficiently severe in one instance to necessitate cessation of dosage on the twenty-eighth day. Interference with bromsulfalein clearance was observed as early as the twelfth day and as late as the fifty-fourth day. It was usually slight and, apart from the instance already noted, was transient.

Malaise, depression, and lassitude: A feeling of malaise and depression was recorded in 19 cases. In one patient the depression was so severe that it was necessary to stop dosage. In the other patients, symptoms were slight and transient. Lassitude was complained of in 15 cases. In one case, the lassitude was so extreme as to necessitate stopping treatment.

Conjunctivitis: Conjunctivitis was observed in 10 cases, mostly in the third and fourth weeks of treatment. The symptoms in all cases were transient, lasting from twenty-four hours to ten days, commonly two to three days. Complaint was made of smarting and watering of the eyes, and slight photophobia. Slight conjunctival congestion was observed.

Exanthemata: Transient rashes were observed in 6 patients, usually in the third and fourth weeks. Usually the rash was morbilliform in character and affected the trunk and limbs chiefly. In 2 cases, itchy lesions of the palms and soles were present. The rash rapidly disappeared after the administration of antihistaminic drugs. The rashes did not recur, despite continued administration of the drug.

Miscellaneous symptoms: Thirst was complained of by one patient and, in 3 female patients, a transient feeling of tightness of the breasts was recorded. No evidence of renal toxicity was observed.

It was noticeable that all toxic symptoms disappeared rapidly following cessation of dosage.

Relationship of Toxicity to Blood Concentrations

The estimated average blood concentrations of Berculon B in patients of all dosage groups is shown in table 1 and the relation of toxicity to the concentration

of drug in the blood in table 3. These demonstrate that there is a reasonable correlation between blood concentrations of Berculon B and the occurrence of toxicity of sufficient severity to necessitate cessation of dosage. At average concentrations of less than 0.25 mg. per 100 ml., 2 patients of 16 had to stop dosage. At concentrations greater than 0.25 mg. per 100 ml. it was necessary to stop dosage in 16 of 24 patients.

Comparison of results obtained at individual hospitals showed a significant difference in the mean blood concentrations and in the incidence of toxicity. This is demonstrated in table 4, where the estimated blood concentration for a dosage of 1.5 mg. per kg. twice daily is recorded with respect to each hospital. The differences in the estimated blood concentrations are significant (greater than twice the standard error) for hospitals A (0.51 mg. per 100 ml.) and B (0.41 mg. per 100 ml.), compared with hospitals C (0.26 mg. per 100 ml.) and D (0.27 mg. per 100 ml.). The possibility existed that there were differences in criteria of toxicity sufficient to stop dosage in the four hospitals or that some environmental factor such as diet, or time of dosage in relation to meals, was responsible for the

TABLE 3

INCIDENCE OF TOXICITY SUFFICIENT TO STOP DOSAGE OF THIOSEMICARBAZONE (BERCULON B) IN RELATION TO BLOOD CONCENTRATION

BLOOD CONCENTRATION OF BERCULON B	NUMBER OF PATIENTS	NUMBER IN WHOM DOSAGE WAS STOPPED	PER CENT IN WHOM DOSAGE WAS STOPPED	
mg. per 100 ml.				
0.10–0.24	16	2	12.5	
0.25–0.39	11	6	54.5	67
>0.4	13	10	77.0	

significant variation. No such obvious difference existed. Further analysis, however, showed that there was a considerable variation between the hospitals in terms of the stage of disease of the patient (table 4). As may be seen in table 4, in hospitals A and B, 12 patients were in an early or intermediate stage of the disease, compared with only one such case in hospitals C and D. Cessation of treatment was necessary in all 3 early cases, in 6 of 10 intermediate cases, and in 9 of 27 advanced cases. However, there is no detectable difference between the blood concentrations in early and intermediate cases at hospitals A and B when compared with advanced cases in the same hospitals. The apparently increased toxicity in early and intermediate cases is probably merely a result of the higher blood concentrations at hospitals A and B, the reason for which remains obscure.

Relation of Blood Concentrations to Dosage

The average blood concentrations of Berculon B of each dosage group and the four-hour increase in blood concentration following each dose are shown in table 1 and figure 1. These show that the blood concentration increases in approximately linear fashion over the range of dosage examined. This is also true of the

rate of increase. However, there was a two- to threefold variation in the blood concentration between persons in any one dosage group. Simmons and his associates (2) suggest that the same is true of thiacetazone. The large variation in the drug concentrations between persons in each group suggests that differences in absorption and metabolism of the drug have a significant effect on the ensuing concentration of drug in the blood.

TABLE 4

ESTIMATED BLOOD CONCENTRATIONS OF THIOSEMICARBAZONE (BERCULON B), RATE OF INCREASE OF BLOOD CONCENTRATIONS, AND INCIDENCE OF TOXICITY SUFFICIENT TO STOP DOSAGE AT FOUR HOSPITALS

HOSPITAL	ESTIMATED BLOOD CONCENTRATION FOR DOSE OF 1.5 MG. PER KG. TWICE DAILY	RATE OF INCREASE PER 1 MG. PER KG. INCREASE IN DOSE	NUMBER OF PATIENTS IN WHOM DRUG WAS STOPPED	NUMBER OF EARLY AND INTERMEDIATE CASES
	mg. per 100 ml.			
A	0.51±0.04*	0.57±0.13	6 of 10	7 of 10
B	0.41±0.02	0.43±0.08	7 of 10	5 of 10
C	0.26±0.02	0.20±0.06	3 of 10	1 of 10
D	0.27±0.02	0.23±0.06	2 of 10	0 of 10

* Standard error.

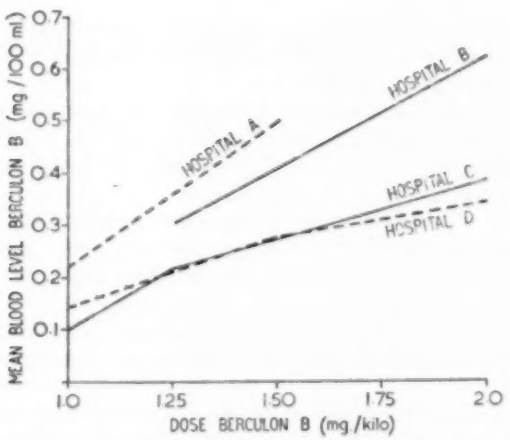

FIG. 1. Mean blood concentration of para-ethylsulfonyl benzaldehyde thiosemicarbazone (Berculon B) in relation to dosage.

It was observed that the blood concentration remains relatively stable throughout treatment in the majority of persons. In only 3 cases was there evidence of accumulation of the drug during the early stages of therapy.

COMMENT

The pattern of toxicity of Berculon B is similar to that of thiacetazone. Gastrointestinal disturbances, anemia, liver dysfunction, malaise, depression, and lassi-

tude, exanthemata, and conjunctival irritation were the main symptoms of toxicity observed in the present trial. However, a detailed comparison of the degree of toxicity of Berculon B and thiacetazone is not easily made. Widely differing opinions have been expressed regarding the toxicity of thiacetazone even when comparable dosage has been used. Analysis of current reports suggests that, with the dosage commonly employed (100 mg. twice daily), toxic manifestations may be expected in approximately 50 per cent of patients and, in 25 per cent, the reactions may be sufficiently severe to necessitate stopping the drug. When 50 mg. twice daily are given, toxic manifestations are uncommon.

From the observations recorded in the present trial, it appears that Berculon B will be well tolerated in the majority of patients provided that concentrations of the drug do not exceed 0.20 to 0.25 mg. per 100 ml. The dosage required to achieve this blood concentration will be approximately 1 mg. per kg., i.e., 50 to 75 mg. twice daily. With this dosage, the drug concentrations in the blood will be higher than those obtained with 100 mg. of thiacetazone twice daily (approximately 0.1 to 0.15 mg. per 100 ml.) and toxicity will probably be less. If Berculon B is given in doses in excess of 1 mg. per kg. twice daily, significant toxicity may be expected.

SUMMARY

The results are recorded of a pharmacologic trial of para-ethylsulfonyl benzaldehyde thiosemicarbazone (Berculon B) in 40 patients, the drug being administered for a period up to eighty-one days.

The drug was administered twice daily in doses which ranged from 1.0 mg. to 2.0 mg. per kg. This range was chosen deliberately as it was considered that such dosage would yield significant information regarding the toxicity of the drug and its relation to dosage.

The pattern of toxicity was similar to that already described in the studies of thiacetazone. Gastrointestinal disturbances and progressive anemia were frequently encountered; and liver dysfunction, malaise, depression and lassitude, exanthemata, and conjunctival irritation also occurred.

Average blood concentrations of the drug increased linearly with dosage but there were large variations between the individual concentrations at any one dosage level.

In concentrations in the blood which did not exceed 0.25 mg. per 100 ml., Berculon B was well tolerated in the majority of patients. Such a concentration is achieved by the administration of approximately 1 mg. per kg., i.e., 50 to 75 mg. twice daily. The concentration in the blood achieved by this dosage is higher than that obtained by the administration of 100 mg. of thiacetazone twice daily (0.1 to 0.15 mg. per 100 ml.).

SUMARIO

Ensayo Farmacológico en Seres Humanos de la Tiosemicarbazona Para-Etilsulfonílica de Benzaldehido (Berculón B)

Preséntanse los resultados obtenidos en un ensayo farmacológico de la tiosemicarbazona para-etilsulfonílica de benzaldehido (Berculón B) en 40 enfermos, a los que se administró la droga durante un período de tiempo que llegó hasta 81 días.

El medicamento fué administrado dos veces diarias a dosis que oscilaban entre 1.0 y 2.0 mg. por kg., escogiéndose esos límites deliberadamente por considerarse que dicha dosificación aportaría datos importantes en cuanto a la toxicidad de la droga y su relación con la posología.

El patrón de la toxicidad fué similar al ya descrito en los estudios de la tiacetazona, droga esta conocida en las E.U.A. con el nombre de amitiozona. Observáronse frecuentemente trastornos gastrointestinales y anemia progresiva, habiendo además disfunción hepática, malestar, depresión y lasitud, exantemas e irritación conjuntival.

Las concentraciones sanguíneas medias aumentaron en proporción lineal a la dosis, pero hubo grandes variaciones entre las distintas concentraciones a cualquier nivel terapéutico dado.

A concentraciones sanguíneas que no excedían de 0.25 mg. por 100 ml., el Berculón B fué bien tolerado por la mayoría de los enfermos. Obtiénense esas concentraciones administrando aproximadamente 1 mg. por kg., o sea, de 50 a 75 mg. dos veces diarias. La concentración sanguínea producida por dicha dosis es más alta que la obtenida con la administración de 100 mg. de tiacetazona dos veces diarias (0.1 a 0.15 mg. por 100 ml.)

RESUME

Essai pharmacologique du para-éthysulfonyl benzaldéhyde thiosemicarbazone, le Berculon B, chez l'homme

Exposé des résultats de l'essai pharmacologique du para-éthylsulfonyl (Berculon B) chez quarante malades; le médicament étant administré pendant une période pouvant atteindre 81 jours.

Ce médicament a été administré deux fois par jour; les doses s'échelonnant entre 1 à 2 mg. par kg., cette gamme a été choisie intentionnellement, car les auteurs considèrent que ces doses donnent des informations valables sur les rapports de la toxicité du médicament et de la dose.

L'expression de la toxicité était semblable à celle antérieurement décrite au cours des études sur la thiacétazone (Connue aux Etats-Unis sous le nom de amithiozone). Les troubles gastro-intestinaux et l'anémie progressive ont été fréquemment observés; la dysfonction hépatique, les malaises, la dépression, la fatigue, l'exanthème et l'irritation conjonctivale se sont également produits.

Les concentrations sanguines moyennes du composé augmentaient en proportion linéaire avec la dose, mais il existait de grandes variations entre les concentrations individuelles quelle que fut la valeur de la dose.

Lorsque les concentrations sanguines, ne dépassaient pas 0,25 mg. par 100 ml. le Berculon B était bien toléré par la majorité des malades. Cette concentration est obtenue par l'administration d'une dose approximative de 1 mg. par kg. c'est dire 50 à 75 mg. deux fois par jour. La concentration sanguine qui est obtenue avec cette dose est plus élevée que celle résultant de l'administration de 100 mg. de thiacétazone deux fois par jour (0,1 à 0,15 mg. pour 100 ml.)

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ISONIAZID IN COMBINED CHEMOTHERAPY OF EXPERIMENTAL TUBERCULOSIS IN MICE[1,2]

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INTRODUCTION

Of a series of reports dealing with the discovery of marked antituberculous activity of isoniazid, those by Grunberg and Schnitzer (1, 3) and Grunberg and associates (2) are based essentially on observations made in mice whose treatment commenced immediately after intravenous inoculation with a virulent human or bovine strain of *M. tuberculosis*. Under these conditions, it was reported that isoniazid in suitable dosage was capable of controlling the infection, the majority of the surviving mice showing no tuberculous lesions and yielding negative subcultures for *M. tuberculosis* from the lung.

The observations reported here deal with the effect of isoniazid, streptomycin, and their combination in experimental tuberculous infection in mice. This investigation was prompted by an earlier observation (5) that, in established seven-day old murine tuberculosis induced by intravenous inoculation with the Ravenel strain, it was impossible to eradicate the infection by means of short-term (twenty-one-day) oral therapy with isoniazid in a dosage of 40 mg. per kg. of body weight. Although these earlier experiments which dealt with delayed chemotherapy did fully confirm the marked superiority of isoniazid over thiosemicarbazone (ethizone) and over streptomycin, some of the apparently healthy isoniazid-treated survivors showed in their lungs small tuberculous lesions and the presence of viable tubercle bacilli which retained their full virulence for guinea pigs.

The purpose of the present study is to investigate whether combined chemotherapy offers any advantage over the use of isoniazid alone and whether there is a marked difference between the effect of early treatment as compared with treatment delayed for seven days after inoculation. The experimental data presented include observations made in mice treated with isonicotinyl-hydrazone of *p*-hydroxy-benzaldehyde,[3] which compound showed in earlier experiments (5) a marked *in vivo* antituberculous activity and low toxicity in mice. This compound is one of a series of compounds synthesized in our laboratories by Dr. A. Zubrys and is designated *compound 327* in the present report.

[1] From the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, Canada.

[2] This investigation was supported by a grant-in-aid from the National Research Council of Canada.

[3] After submitting this manuscript, the following two studies came to our attention: Domagk and his associates (11) included the above compound among a series of isonicotinyl-hydrazones described as antituberculous. An interesting carboxylic acid derivative of this same compound was recently described by Levaditi and Vaisman (12).

METHODS

Female albino mice of an inbred (Connaught) strain, weighing 16 to 18 gm., were inoculated intravenously with the Ravenel strain of *M. tuberculosis*.[4] The culture was grown for nine days in a modified Dubos medium. The infective dose was adjusted to correspond to 0.2 mg. of moist weight of growth contained in 0.4 ml. of medium. This dose killed all control mice between the seventeenth and twenty-second days.

Streptomycin sulfate was injected subcutaneously once daily for twenty-four of a total of twenty-eight days. Isoniazid and the isonicotinyl hydrazone of *p*-hydroxy-benzaldehyde (compound 327) were administered orally admixed to the standard diet which consisted of ground fox chow. Dosages are expressed in mg. per kg. of body weight and are calculated on the basis of an average food intake of 4 gm. per 20-gram mouse.

TABLE 1

EARLY CHEMOTHERAPY OF MURINE TUBERCULOSIS

Infection: Intravenous Inoculation with Ravenel Strain of *M. Tuberculosis*

Treatment: 28 Days. Start: On Day of Inoculation*

TREATMENT		SURVIVORS, FORTY-SECOND DAY	EXAMINATION OF LUNGS					
			Subcultures for *M. Tuberculosis* on Löwenstein Medium					Amount of Gross Tuberculosis
Drug	Mg. per Kg.		Positive Streak Cultures†	Lung Suspension Colony Counts‡				
Isoniazid	40	10/10	6/9	8	18	6	100 25	0.3+
Streptomycin	100	10/10	10/10	∞§	∞	∞	∞ ∞	2+
Isoniazid plus streptomycin	40 / 100	10/10	1/10	0	0	1	0 0	0
Compound 327	200	10/10	7/9	10	6	17	9 27	0.1+
Controls	—	0/10						3+

* See methods.

† Only those tests are recorded which were not vitiated by contaminants.

‡ Each figure represents count derived from lung of individual mouse.

§ Innumerable colonies.

A total of 106 mice was used for the experiment which comprised eleven groups of mice. *Groups I* to *IV* underwent early chemotherapy, commencing on the day of inoculation (table 1); whereas *Groups V* to *IX* received delayed chemotherapy, starting on the seventh day after infection (table 2). Ten infected untreated mice served as controls for both parts of the experiment. Six supplementary untreated control mice were killed on the seventh day after inoculation.

Although medicated diet was started immediately after inoculation in early treatment (table 1), the first streptomycin injection was not given until eighteen hours later.

As indicated in table 1, the following forms of chemotherapy were used: isoniazid, streptomycin, isoniazid plus streptomycin, and compound 327. These same treatments were

[4] Obtained through the courtesy of William Steenken, Jr., Trudeau Laboratory, Trudeau, New York.

used for delayed chemotherapy (table 2). The combination of all three drugs, isoniazid plus streptomycin plus compound 327, was used for delayed treatment only.

Chemotherapy was maintained for twenty-eight days. After two weeks of rest, all of the surviving treated mice were sacrificed and examined for gross tuberculous lesions. The scheme used for recording the amount of gross tuberculosis (tables 1 and 2) is that proposed by Youmans (6). Histologic sections of lungs, liver, and spleen were prepared. The cultivation of tubercle bacilli from the lungs was effected (*a*) by streaking the freshly cut surface of a lobe across a Löwenstein-Jensen slant, and (*b*) by grinding a second lobe in a ground glass tissue grinder and suspending the material in Tween[5]-albumin medium

TABLE 2*

DELAYED CHEMOTHERAPY OF MURINE TUBERCULOSIS

Infection: Intravenous Inoculation with Ravenel Strain of *M. Tuberculosis*

Treatment: 28 Days. Start: On Seventh Day after Inoculation

TREATMENT		SURVIVORS FORTY-NINTH DAY	EXAMINATION OF LUNGS						
			Subcultures for *M. Tuberculosis* on Löwenstein Medium						Amount of Gross Tuberculosis
Drug	Mg. per Kg.		Positive Streak Cultures	Lung Suspension Colony Counts					
Isoniazid	40	10/10	6/9	100	66	75	80	4	0.7+
Streptomycin	100	8/10	8/8	∞	∞	∞	∞	∞	2.6+
Isoniazid plus streptomycin	40 / 100	10/10	7/9	95	38	7	9	27	0.5+
Compound 327	200	10/10	5/8	100	60	16	6	3	0.4+
Isoniazid plus streptomycin plus compound 327	40 / 100 / 200	10/10	1/10	0	0	0	0	1	0
Controls†	—	0/10							3+

* All footnotes of table 1 apply also to table 2.

† Controls identical with those recorded in table 1.

containing 20 units of potassium penicillin G per ml. Of this suspension, an aliquot part, corresponding to one-fiftieth of the lobe, was seeded on a large Löwenstein-Jensen slope. For virulence tests the same suspension was used for subcutaneous inoculation into tuberculin-negative guinea pigs.

RESULTS

As the great majority of the treated mice survived the test period, a comparison of the effects of different forms of chemotherapy had to be made on the basis of bacteriologic and histologic findings.

[5] Tween 80 is the proprietary name for the polyoxyethylene derivative of Sorbitan monooleate, obtainable from the Atlas Powder Co., Wilmington, Delaware.

Early chemotherapy: In early chemotherapy (table 1), only some of the mice treated with isoniazid showed on gross examination a few small tuberculous lesions in the lungs, whereas in the group treated with streptomycin the lungs of all 10 mice were markedly enlarged and contained numerous well-defined tuberculous foci in all of the lobes. The mice which received compound 327 presented essentially the same favorable picture as those treated with isoniazid. The only form of early treatment which resulted in the complete absence from the lungs of visible tuberculous lesions is the combination of isoniazid and streptomycin. Only one of 10 mice thus treated yielded a positive culture from the lungs, showing one single colony of *M. tuberculosis*. In the same group, colony counts made on lung suspensions revealed the presence of viable tubercle bacilli in only one of the 5 mice tested.

Delayed chemotherapy: With delayed chemotherapy (table 2), the lungs of mice which were treated with streptomycin showed a more advanced stage of tuberculous infection than those of the corresponding groups which received early treatment. The post-mortem findings in the two groups of mice on isoniazid and on compound 327, respectively, did not markedly differ from those recorded for the mice which received early chemotherapy with the same drugs, although the colony counts were, on the average, higher for the delayed treatment.

A more distinct difference between the effects of early and delayed chemotherapy became apparent, however, with the combination of isoniazid and streptomycin. With this form of delayed treatment, the positive streak cultures and the colony counts revealed the persistence of viable tubercle bacilli in the lungs of the majority of the mice. Only the combination of the three drugs, as used for the last group of treated mice (table 2), proved sufficiently effective to result in the complete absence of visible lesions from the lungs and subcultures negative for tubercle bacilli for 9 of 10 mice. This finding was confirmed by negative subcultures from lung suspensions obtained for 4 of the 5 mice tested.

Histologic sections: Marked changes in the lungs and spleen were limited to the following groups of mice: untreated infected controls, pretreatment controls sacrificed seven days after inoculation, and all mice which had early or delayed streptomycin therapy.

The lungs showed more or less marked obliteration of alveolar spaces and the presence of numerous tubercle bacilli. In the spleen the lymphatic nodules were reduced and fragmented by tuberculous lesions which contained numerous acid-fast bacilli as described for the experimental infection of mice produced by the H37Rv strain (7).

The lungs and spleen of mice treated with isoniazid, compound 327, or the combination of these drugs with streptomycin failed to show the above-mentioned changes. Acid-fast bacilli were scarce, if present at all.

In the liver the only unexpected changes were those suggestive of beginning fatty degeneration, noticeable in mice which had early or delayed chemotherapy with the combination of isoniazid plus streptomycin or delayed treatment with compound 327.

Guinea pig inoculations: Eleven guinea pigs were inoculated subcutaneously

into the groin with lung suspensions from mice representing the various treated groups (tables 1 and 2). Only one guinea pig failed to develop a lesion at the site of inoculation and remained tuberculin-negative to 1 mg. of Old Tuberculin (OT) as of the eighty-fourth day. This guinea pig had been inoculated with lung material from a mouse treated with the combination of the three drugs (table 2).

The remaining guinea pigs, which had received lung material from mice of the other groups, developed tuberculin sensitivity and marked local lesions with involvement of the inguinal nodes. The final outcome of these tests will be recorded in a later report.

DISCUSSION

The observations presented confirm the previously reported (3, 4) superiority of isoniazid over streptomycin in the short-term treatment of experimental tuberculosis in mice induced by the Ravenel strain. This conclusion applies to the early as well as to the delayed treatment of the infection, and is based on results obtained with subcultures from the lungs supplemented by colony counts. It is supported further by gross and microscopic examination of the lungs.

Bernstein and co-workers (4) reported the superiority of isoniazid over streptomycin in the delayed treatment of the murine infection, although these writers did not deal with the important question of the possible presence in the surviving mice of residual tuberculous infection.

The conditions of the present experiment, in which the effect of isoniazid falls short of eradicating the infection from the lung, lend themselves to the quantitative study of the effect of drug combinations.

With early treatment (table 1), the combination of isoniazid and streptomycin is considerably more effective than isoniazid alone and is capable of reducing the infection to the point where subcultures from the lungs of the majority of the mice fail to reveal the presence of viable tubercle bacilli. A similar marked superiority of the combination of streptomycin and isoniazid was reported by Goulding and Robson (8) who made a study of the effect of isoniazid in the control of experimental corneal tuberculosis in mice. These writers dealt mainly with the early treatment of the infection. Their observations made on the effect of chemotherapy in the established ocular infection seem to support the present findings, which indicate that in delayed treatment (table 2) some of the advantage of the combination of isoniazid and streptomycin over the use of isoniazid alone is lost.

While the present investigation was proceeding, Joiner and associates (9) published clinical observations which suggest that the combination of isoniazid and streptomycin in the treatment of pulmonary tuberculosis gives results which are superior to those obtained with isoniazid alone.

The observed persistence of viable tubercle bacilli in the lungs of mice which received twenty-eight days' treatment with isoniazid does not lessen the efficacy of this drug as a very powerful antituberculous agent. But it emphasizes the difficulty of eradicating an overwhelming experimental infection by means of a short-term treatment with isoniazid alone.

It appears to be of some interest that in using delayed chemotherapy (table 2)

it was impossible, under the conditions of the present experiment, to obtain negative subcultures from the lungs of mice which received the combination of isoniazid and streptomycin. In contrast, when the same two drugs were used in association with compound 327, the majority of the mice failed to show the presence of viable tubercle bacilli in the lungs. Although it seems possible that the same favorable result could be obtained in mice by using larger doses of isoniazid in combination with streptomycin, the limited tolerance of man for isoniazid (10) suggests that it may be worth while to explore further the possibility of combining streptomycin with an antituberculous drug which is of lower toxicity to man than isoniazid.

The choice of compound 327 for inclusion in the above experiment was based on results of screen tests in which a number of isonicotinylhydrazones of substituted benzaldehydes were tested in the established tuberculous infection in mice. Of a number of compounds of this type, which show low chronic toxicity in normal mice, compound 327 proved to be one of the most active. A detailed report of these tests will be published.

In assessing the possible toxic effects of the drug treatments, one has to take into account that dosages far in excess of those applicable for man were employed for short-term chemotherapy of the experimental infection. It seems likely that the observed liver changes, suggestive of fatty degeneration, which occurred in some of the mice treated with isoniazid plus streptomycin or with compound 327 are the combined effect of drug action plus infection. This view is supported by the observation that neither the infection by itself nor the drug treatment alone produced similar changes, which latter point was ascertained by chronic toxicity experiments. The occurrence of the above-described liver changes in some of the treated mice was not sufficiently consistent to permit final conclusions, but it emphasizes the necessity of extending the study of chronic toxicity from the normal to the tuberculous animal.

SUMMARY

Chemotherapeutic tests in mice infected with the Ravenel strain of *M. tuberculosis* confirmed the marked superiority of isoniazid over streptomycin, both for early treatment as well as for delayed chemotherapy started seven days after inoculation.

The combination of isoniazid with streptomycin proved more effective than isoniazid alone and resulted, with early treatment, in the complete absence from the lungs of visible tuberculous lesions which gave subcultures negative for tubercle bacilli in the majority of the mice.

With delayed treatment, the combination of isoniazid and atreptomycin, although more effective than isoniazid alone, failed to eliminate the presence of viable tubercle bacilli from the lungs, whereas a combination of isoniazid plus streptomycin with the isonicotinyl hydrazone of *p*-hydroxy-benzaldehyde prevented the development of gross lesions and resulted in the absence of viable tubercle bacilli from the lungs of the majority of the mice.

Although the antituberculous activity of the isonicotinyl hydrazone of *p*-

hydroxy-benzaldehyde is below that of isoniazid, its much lower chronic toxicity for mice prompted its choice for investigative work.

SUMARIO

La Isoniacida en la Quimioterapia Combinada de la Tuberculosis Experimental en los Ratones

Pruebas quimioterapéuticas ejecutadas en ratones infectados con la cepa Ravenel del *M. tuberculosis* confirmaron la decidida superioridad de la isoniacida sobre la estreptomicina tanto para el tratamiento temprano cuanto para la quimioterapia retardada iniciada a los siete días de la inoculación.

La combinación de la isoniacida con la estreptomicina resultó más eficaz que la isoniacida sola y fué seguida, en casos de tratamiento temprano, de la ausencia absoluta de lesiones tuberculosas visibles en el pulmón que dió subcultivos negativos para la mayoría de los ratones.

En los casos de tratamiento retardado, la combinación de isoniacida con estreptomicina, aunque más eficaz que la isoniacida sola, no eliminó la presencia en el pulmón de bacilos tuberculosos viables, en tanto que una combinación de isoniacida y estreptomicina con la hidrazona isonicotínila del *p*-hidroxi-benzaldehido impidió la formación de lesiones macroscópicas e hizo desaparecer los bacilos tuberculosos viables de los pulmones de la mayoría de los ratones.

Aunque la actividad antituberculosa de la hidrazona isonicotínila del *p*-hidro-ixbenzaldehido es inferior a la de la isoniacida, su toxicidad crónica mucho menor para los ratones motivó su selección para trabajos de investigación.

RESUME

L'Isoniazide dans la chimiothérapie combinée de la tuberculose expérimentale de la souris

Des tests chimiothérapeutiques effectués chez des souris inoculées avec la souche Ravenel du *M. tuberculosis* ont confirmé la supériorité de l'isoniazide sur la streptomycine, aussi bien pour le traitement précoce que pour la chimiothérapie retardée, instaurée sept jours après l'inoculation.

L'association de l'isoniazide et de la streptomycine s'est montrée plus efficace que le traitement par l'isonizaide seule et, dans le cas d'un traitement précoce, elle a eu pour résultat l'absence complète de lésions tuberculeuses visibles dans le poumon et celui-ci a donné des subcultures négatives chez la plupart des souris.

Dans le cas d'un traitement retardé, l'association isoniazide-streptomycine, bien que plus efficace que l'administration de l'isoniazide seule, n'a pas réussi à éliminer la présence de bacilles tuberculeux viables dans le poumon, tandis que l'association isoniazide-streptomycine plus l'hydrazone isonicotinylique de *p*-hydroxy-benzaldéhyde a prévenu le développement de lésions macroscopiques et a permis de démontrer l'absence de bacilles tuberculeux viables dans les poumons de la majorité des souris.

Bien que l'activité antituberculeuse de l'hydrazone isonicotinylique de *p*-hydroxy-benzaldéhyde soit inférieure à celle de l'isoniazide, le degré de sa toxicité chronique chez la souris, qui est beaucoup plus bas, l'a fait adopter pour les travaux d'investigation.

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A STUDY OF THE EFFECT OF ALCOHOLS ON TUBERCLE BACILLI AND OTHER BACTERIA IN SPUTUM[1]

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INTRODUCTION

In 1947, Smith (1) described experiments indicating that ethyl alcohol is an effective agent for killing tubercle bacilli in sputum when the sputum is smeared on coverslips and is pre-treated with 0.25 per cent of sodium hydroxide. In the present laboratory, Smith's observations concerning the effectiveness of the ethyl-alcohol–sodium hydroxide treatment, using sputum spread thinly on glass rods (simulating thermometers) has been confirmed. Because of almost universal availability of ethyl alcohol or isopropyl (rubbing) alcohol in hospitals, public health nurses' bags, and homes, it was believed that it would be worth while to make further studies of the effects of these agents on tubercle bacilli and other pathogenic bacteria, especially in larger quantities of sputum, i.e., in sputum cups.

MATERIALS AND METHODS

Sputum: One hundred and seventeen specimens of sputum obtained from 117 patients were used. The specimens were less than twenty-four hours old and were obtained from patients who had pulmonary tuberculosis. Specimens were taken only from patients who were not receiving chemotherapy and in whose sputum acid-fast bacilli were readily demonstrable in Ziehl-Neelsen–stained smears.

Each specimen was transferred to a sterile Petri dish and was additionally contaminated with 0.1 ml. of a 24-hour broth culture of *Corynebacterium diphtheriae*.

Cultures: Löwenstein-Jensen-Holm medium was used for the cultivation of tubercle bacilli. Of an aqueous solution of penicillin G potassium (containing 1,000 units per ml.), 0.1 ml. was placed on the surface of each slant within 24 hours before inoculation (2). Penicillin was not used on the controls prepared from sputum treated with sodium hydroxide.

Cultures for *Streptococci*, *Staphylococci*, and diphtheria bacilli were made on heart-infusion-dextrose (0.5 per cent)-agar (Difco) slants, on which had been placed approximately 0.05 ml. of defibrinated rabbit's blood. In the first experiments, Löffler's slants were used for the cultivation of *C. diphtheriae*, but this was discontinued when it was found that heart-infusion-agar slants served as well for purposes of isolation only. When the heart-infusion-agar slants showed any growth, smears and broth subcultures were prepared. The broth cultures were incubated and blood-agar plates were poured. Random samples of the colonies which grew on the blood-agar plates were picked and identified as far as genus.

Plan of Study

Control experiments (for detecting the presence of the test organism in untreated sputum):

a. One loopful (4 mm.) of untreated sputum from each specimen was streaked on the

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surface of slants of Löwenstein-Jensen-Holm medium prepared with penicillin, as described above, and also on the surface of heart-infusion-agar slants.

b. One ml. of 4 per cent sodium hydroxide was added to 1 ml. of the raw sputum in a test tube and was allowed to stand with occasional shaking at room temperature for thirty minutes. After neutralization with 2N hydrochloric acid, 0.1 ml. was streaked on slants of Löwenstein-Jensen-Holm medium.

Tests:

Series A (*alcohol-treated sputum*): Five milliliters of alcohol in various concentrations were added to 1 ml. of sputum in test tubes and allowed to stand for various intervals (*see* Results). A tough, gummy coagulum formed immediately. This was removed with sterile applicator sticks and suspended in 5 ml. of sterile water to dilute the alcohol. One loopful of the coagulum was streaked on slants of the medium. With liquid specimens in which no large coagula were formed by the addition of alcohol, 1 ml. of sediment was pipetted into a tube of sterile water, and 0.2 ml. of this suspension was inoculated on the surface of the slants.

Series B (*coagula plus shaking*): The distilled water suspensions prepared in *Series A* were shaken for ten minutes in a mechanical paint shaker and 0.2 ml. of each was then inoculated on the slants.

Series C (*alcohol-sputum-shaking treatment*): This procedure was the same as described in *Series A* except that the alcohol-sputum mixtures were placed immediately (within sixty seconds) in the paint-shaking machine for the time intervals of disinfection. After shaking, the coagula which was produced, or 1 ml. of each of the suspensions, were placed in 5 ml. of sterile water as described in *Series A*, and 0.2 ml. of these mixtures was streaked on the slants.

Series D (*coagula of Series C plus shaking*): The coagula (or suspensions) and distilled water mixtures prepared in *Series C* were shaken in the paint-shaking machine for ten minutes, and 0.2 ml. of each suspension was placed on the surfaces of the slants.

All cultures were incubated at 37°C. Löffler's slants and heart-infusion-agar slants were kept for one week unless growth occurred before that time. Subcultures were made from tubes which showed evidence of growth, and genus identity was established.

Tubes of Löwenstein-Jensen-Holm medium were slanted in a nearly horizontal position for forty-eight hours at 37°C., after which the tubes were kept upright. Routine gross examination of the tubes was performed weekly for six weeks. Gross readings were confirmed by microscopic examination of stained smears. Questionable growth was confirmed by the Tuberculosis Laboratory of the Communicable Disease Center.

RESULTS

The results of tests which yielded viable tubercle bacilli are shown in table 1. In table 2 the data for *Staphylococci*, *Streptococci*, and diphtheria bacilli are given.

It is evident that, when coagula are formed by the addition of ethyl or isopropyl alcohol to sputum, tubercle bacilli can be recovered from a large number of them (*see A*, table 1). Even after the coagula formed in *Series A* were broken up with water in the paint shaker, a high percentage of the cultures yielded viable tubercle bacilli, *Staphylococci*, *Streptococci*, and diptheria bacilli (*see B*, tables 1 and 2). This seems to support the widely held belief that sputum coagula protect organisms occluded within them. Experimental data in support of this old idea were not found in the literature examined.

When the mixture of alcohol and sputum was shaken violently immediately after the mixture was made, the test organisms were nearly always killed, even by a two-minute exposure and even though a small amount of coagulum was formed (*C*, tables 1 and 2).

TABLE 1

EFFECT OF ALCOHOLS ON THE CULTIVATION OF TUBERCLE BACILLI FROM SPUTUM WITH AND WITHOUT MECHANICAL AGITATION

SUBSTANCES AND TIME	NUMBER OF SPECIMENS TESTED	CONTROLS* (UNTREATED)			CONTROLS* (NaOH-TREATED)			A†			B†			C†			D†		
		Positive	Contaminated‡	Negative	Positive	Contaminated‡	Negative	Positive	Contaminated‡	Negative	Positive	Contaminated‡	Negative	Positive	Contaminated‡	Negative	Positive	Contaminated‡	Negative
Two-minute treatment																			
Ethyl alcohol, 100 per cent	13	9	3	1	9	1	3	8	3	2	5	6	2	1	0	12	—	—	—
Ethyl alcohol, 95 per cent	14	12	2	0	11	2	1	10	2	2	11	3	0	—	—	—	—	—	—
Ethyl alcohol, 80 per cent	14	12	1	1	13	0	1	7	3	4	11	0	3	0	0	14	—	—	—
Isopropyl alcohol (rubbing), 70 per cent	14	11	3	0	14	0	0	8	0	6	8	0	6	2	0	12	2	0	12
Ten-minute treatment																			
Ethyl alcohol, 100 per cent	12	8	4	0	8	0	4	8	2	2	6	4	2	0	0	12	0	0	12
Ethyl alcohol, 95 per cent	13	11	2	0	13	0	0	6	1	6	2	6	5	0	0	13	0	0	13
Ethyl alcohol, 80 per cent	13	11	2	0	13	0	0	8	1	4	9	2	2	1	0	12	1	0	12
Isopropyl alcohol (rubbing), 70 per cent	12	11	1	0	12	0	0	6	2	4	8	0	4	0	0	12	0	2	10
Isopropyl alcohol (rubbing), 50 per cent	12	10	2	0	10	0	2	7	2	3	7	1	4	0	0	12	0	2	10

* Controls (untreated and NaOH-treated): Tests for recovery of tubercle bacilli from sputum.

† A: Sputum treated with alcohol—no agitation.
B: Alcohol coagulum formed in *A* broken up in paint shaker.
C: Sputum agitated with alcohol in paint shaker—minimal coagulation.
D: Alcohol coagulum formed in *C* broken up in paint shaker.

‡ Contaminated—Fungi and bacteria other than tubercle bacilli present. These tubes negative for tubercle bacilli.

The experiments in *Series D* were included to determine whether the minimal coagulum formed by alcohol-shaken sputum would yield viable organisms when the coagulum was broken up. Only occasionally were viable organisms recovered.

There were no significant differences between the results obtained with ethyl alcohol and those obtained with isopropyl alcohol.

TABLE 2

EFFECT OF ALCOHOLS ON THE CULTIVATION OF STAPHYLOCOCCI, STREPTOCOCCI, AND DIPHTHERIA BACILLI FROM SPUTUM, WITH AND WITHOUT MECHANICAL AGITATION*

SUBSTANCES TESTED AND TIME APPLIED	NUMBER OF SPECIMENS	TUBES YIELDING CULTURES OF											
		Staphylococci				*Streptococci*				Diphtheria bacilli			
		Controls	B	C	D	Controls	B	C	D	Controls	B	C	D
Two minutes:													
Ethyl alcohol, 100 per cent	13	11	9	0	—	13	11	0	—	13	13	0	—
Ethyl alcohol, 95 per cent	14	13	10	—	—	10	10	—	—	14	13	—	—
Ethyl alcohol, 80 per cent	14	14	7	0	—	14	8	0	—	14	10	0	—
Isopropyl alcohol (rubbing), 70 per cent	14	14	5	0	0	11	4	0	0	14	4	0	0
Ten minutes:													
Ethyl alcohol, 100 per cent	12	12	9	1	0	12	9	1	0	12	10	1	0
Ethyl alcohol, 95 per cent	13	13	8	0	1	13	7	0	0	13	8	1	0
Ethyl alcohol, 80 per cent	13	11	3	0	0	12	9	0	4	13	7	1	3
Isopropyl alcohol (rubbing), 70 per cent	12	12	5	0	0	12	9	0	0	12	6	0	0
Isopropyl alcohol (rubbing), 50 per cent	12	10	9	0	0	11	9	0	2	12	8	0	1

* See footnotes to table 1.

DISCUSSION

These experiments indicate that, unless special and unusual precautions are taken, neither ethyl nor isopropyl alcohol may be relied upon to kill the organisms studied in this investigation when the bacteria are in sputum. Experiments published elsewhere (3) show that this also obtains, although to a lesser extent, even when the sputum is present only as a thin film such as adheres to an oral thermometer. That the *Staphylococci*, *Streptococci*, and diphtheria bacillus are

not per se resistant to these alcohols is made clear by another series of experiments (4), in which exposure of these "naked" organisms, i.e., pure cultures suspended in broth, to the same alcohols in the same concentrations resulted in complete destruction in less than one minute. No experiments on pure cultures of tubercle bacilli were made in the present study, but results obtained elsewhere (1) indicate that the organisms are highly susceptible to ethyl alcohol when in the "naked" state.

As noted in the present experiments, when alcohol was added to sputum (except the very liquid specimens), a thick, white coagulum was produced. The addition of alcohol to the liquid specimens produced a flocculent precipitate which settled to the bottom of the tube. This precipitate yielded a much smaller percentage of cultures positive for the test organisms before and after violent mechanical agitation in the paint shaker than did the coagula produced from thick, viscous specimens of sputum. However, the occasional positive cultures obtained from the treated liquid specimens suggest that the alcohols tested are not reliable as disinfectants, even when used upon such small amounts of sputum as may adhere to a thermometer, a bronchoscope, or other instrument contaminated with sputum.

Previous studies by Smith (1) suggest that ethyl alcohol, in commonly used concentrations, is an effective disinfectant against tubercle bacilli in smears of sputum. One major difference between the present study and the study of Smith is that in the present study the sputum was not decontaminated with sodium hydroxide before or after disinfection. Studies in this laboratory show that *Mycobacterium tuberculosis* in thin or thick films of sputum *untreated with NaOH* are not as readily killed by ethyl alcohol as the report by Smith (1) would indicate.

SUMMARY

Ethyl alcohol and isopropyl alcohol ("rubbing alcohol") in 70 per cent concentration are not effective as disinfectants against tubercle bacilli and other bacteria in sputum.

Ethyl alcohol and isopropyl alcohol ("rubbing alcohol") in 70 per cent concentration are partly effective when they are brought into close contact with the tested organisms, as when the sputum and disinfectant are agitated together violently *before* the sputum has time to coagulate significantly.

Sputum (especially viscous sputum) gives considerable protection against alcohol to organisms present in it unless unusual precautions are taken to prevent early coagulation of the sputum.

SUMARIO

Estudio del Efecto de los Alcoholes sobre las Bacterias del Esputo

El alcohol etílico y al alcohol isopropílico ("alcohol de friega") a una concentración de 70 por ciento no son eficaces como desinfectantes contra las bacterias contenidas en el esputo.

El alcohol etílico y el isopropílico a una concentración de 70 por ciento son parcialmente eficaces cuando se ponen en íntimo contacto con los microorganismos ensayados, como su-

cede cuando se agitan juntos violentamente el esputo y el desinfectante *antes* de que el esputo haya tenido tiempo de coagularse de veras.

El esputo (en particular el viscoso) proporciona considerable protección contra el alcohol a los microbios que contiene a menos que se tomen precauciones exageradas para impedir la coagulación temprana del esputo.

RESUME

Une étude sur l'effet des alcools sur les bactéries des crachats

L'alcool éthylique et l'alcool isopropylique ("rubbing alcohol") à 70%, ne sont pas efficaces comme désinfectants contre les bactéries contenues dans les crachats.

L'alcool éthylique et l'alcool isopropylique (rubbing alcohol) à 70%, sont partiellement efficaces quand ils sont mis en contact direct avec les organismes soumis au test, ainsi que lorsque le crachat et le désinfectant sont agités fortement ensemble *avant* que le crachat ait eu le temps de coaguler de façon appréciable.

Le crachat (en particulier le crachat visqueux) offre une protection considérable contre l'action de l'alcool sur les organismes qu'il renferme, à moins que des précautions particulières ne soient prises pour prévenir la coagulation précoce du crachat.

Acknowledgments

The authors are indebted to Miss Virginia Wilson, Miss Anne Barton, and Mr. William Ellis for aiding in carrying out the technical aspects of this study, and to Dr. Robert Patnode for supplying Löwenstein-Jensen-Holm medium and checking doubtful cultures of tubercle bacilli. Specimens of sputum were obtained through the kind cooperation of Dr. Edwin Rapp, Veterans Administration Hospital No. 48, Atlanta, Georgia, and Miss Vivien O'Brien, Lawson Veterans Hospital, Chamblee, Georgia.

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STUDIES ON THE FRACTIONATION OF TUBERCULIN AND JOHNIN[1]

I. Fractionation of Tuberculin Proteins

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INTRODUCTION

Since the inception of the United States Department of Agriculture Bovine Tuberculosis Eradication Program in this country in 1917, the incidence of tuberculosis in cattle has been reduced from approximately 5 per cent to less than 0.2 per cent (1). The diagnostic test used since 1936 is the intradermal injection of Bureau of Animal Industry intradermal strength tuberculin (BAI regular tuberculin), a concentrated filtrate of human-type tubercle bacilli culture grown on synthetic medium (2). Animals which are classed as reactors are condemned and slaughtered in government-inspected establishments and careful post-mortem examinations are made by government veterinary inspectors. At the present time, however, tuberculous lesions cannot be found on post-mortem examination in approximately one-half of the animals condemned (3). Although many of these no-visible-lesions (NVL) cases are probably due to incipient tuberculosis, some proportion of them are undoubtedly due to cross-reactions of tuberculin with heterologous sensitization due to Johne's bacilli, avian tubercle bacilli, or closely related acid-fast organisms (4, 5).

Johne's disease, paratuberculosis, is a chronic enteritis caused by the organism *Mycobacterium paratuberculosis* (6). The infection occurs principally in cattle but is also found in sheep, goats, and deer. Symptoms of the disease are emaciation and diarrhea. Johne's disease is estimated to cause an annual loss in cattle valued at approximately $500,000, and evidence indicates that the disease is widespread and increasing. Control of Johne's disease has been effected to some extent by detecting the infection by means of an intrademal test similar to that used for the detection of tuberculosis (7). Animals which give a positive reaction to this test may then be condemned or isolated. The reagent used for the skin test, BAI regular johnin, is simply a concentrated filtrate of Johne's bacilli cultures (8). It is not completely satisfactory because it is not specific for Johne's disease (5) and it is not uniform from one batch to another.

Baisden and co-workers reported on some rather unpromising attempts to improve johnin and tuberculin by various physical, chemical, and biologic treatments (9).

Further work on the fractionation of tuberculin and johnin has been carried

[1] From the Allergen Research Division, Bureau of Agricultural and Industrial Chemistry, and the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington 25, D. C.; and the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Auburn, Alabama.

out in order to try to obtain materials which are more specific than the regularly produced tuberculins and johnins. A satisfactory preparation should give positive reactions with homologously sensitized animals but little or no reaction with heterologously sensitized animals. The experiments on tuberculin described in papers I and II of this series, and on johnin described in paper III, were carried out during the period 1947 through 1951.

PLAN OF INVESTIGATION

Method of Testing

To determine whether the fractions obtained in this investigation were more specific than BAI regular tuberculin, intradermal tests were made on two groups of 10 cattle each, one group having been sensitized to *Mycobacterium tuberculosis* var. *bovis* and the other to *M. paratuberculosis*. The animals were sensitized at least a month before initiation of the tests by intraperitoneal injection of 3 gm. of the appropriate heat-killed bacilli suspended in mineral oil. Sixteen materials, including a control (regular tuberculin), were tested at a time.

The response–concentration relationship was determined by testing each of the 16 materials at four concentration levels based on their nitrogen contents. The levels selected for all materials depended upon the degree of sensitivity of the cattle, higher levels being selected when the degree of sensitivity was low rather than high, as determined by preliminary tests. The relative levels of an unknown material and the regular tuberculin were selected so that as nearly as possible the same range of response would be covered (figure 1).

Regular BAI tuberculin usually contains approximately 2,300 γ of nitrogen per ml. in its undiluted form. Most of this nitrogen is inactive. Comparisons with unknowns were made on a nitrogen basis, however, because the nitrogen content of various lots of the regular product is slightly different. Moreover, it was assumed from the results of previous work (unpublished) that the same proportion of inactivity of the regular product would be manifested on both homologous and heterologous sensitizations. Comparison of the regular and fractionated tuberculins on a nitrogen basis did not in itself show the fractionated products to possess more specific potencies simply because of the removal of the extraneous or inactive nitrogen. This is shown in the present study by the fact that certain fractionated products were found to possess less specific potencies than the regular material, their potencies on both sensitizations still being fairly high (figure 1).

In expressing the results of the fractionations, the percentage of the total nitrogen of the culture filtrate which is contained in a fraction is given. This was done to indicate the efficiency of the fractionating process rather than to indicate interest in the efficiency of the various types of nitrogen present in the original material. It is admitted that the percentage of the total *active* nitrogen which was present in a fraction was the point of real interest in considering efficiency of a procedure, but, since an accurate estimation of the total active nitrogen is practically impossible, the percentage of the total nitrogen is substituted.

Since various areas of the bovine skin differ in sensitivity to tuberculin (10), four areas were defined on each animal. The 64 injections were placed so there would be 4 materials or 16 injections in each of the four areas on each animal: *area 1*, both sides of the neck; *area 2*, high on both sides; *areas 3* and *4*, on both sides below *area 2*. These areas are shown in a paper by Wadley (11). The four concentration levels of each material were injected in adjacent sites but in random order.

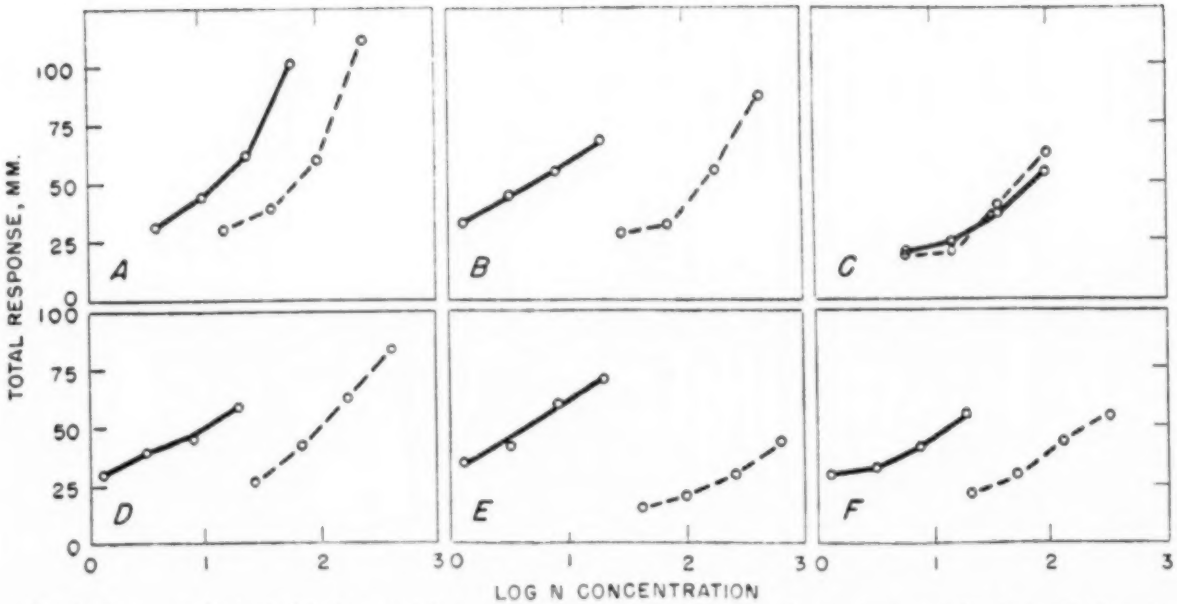

FIG. 1. Responses obtained with six materials tested on one group of cows sensitized to human-type tubercle bacilli (homologous sensitization) and another group sensitized to Johne's bacilli (heterologous). Solid line—homologous; dashed line—heterologous.

Materials: *A*—Bureau of Animal Industry regular tuberculin; *B*—Acid-fractionated material; *C*—Dialysate; *D*—Precipitated from phenol solution at 25 per cent alcohol; *E*—Precipitated from supernatant solution of *D* at 60 per cent alcohol; *F*—Precipitated from concentrated supernatant solution of *E* by addition of ether.

In each group of animals, the 16 materials were randomized with respect to site of injection in such a way that every material was paired with each other material at least twice. The incomplete block plan mentioned by Wadley was used (11, 12). This reduced the error due to differences in sensitivity of the four areas. The skin thickness at the site of injection was measured before injection and the increase due to the injection was determined forty-eight hours later. Skin thickness measurements were made with the dermal thickness gage described by Kummer and Johnson (13).

The responses were analyzed statistically by the method described by Wadley (11, 12) to obtain the potency of each material based on regular tuberculin as unit potency, the regular having been tested on a nitrogen basis. The potency values were then corrected for differences in nitrogen content among the samples. The specificity was calculated as the ratio of the homologous to the heterologous potency. Usually a potency of approximately 2 and a specificity of approximately 3 were required for statistical significance (19:1). For high significance (99:1), these values had to be approximately 3 and 4, respectively. Potency or specificity of samples described as high or low will refer to comparison on a nitrogen basis with BAI regular tuberculin unless otherwise stated.

In many tests the numerical value calculated for the specificity of certain fairly specific materials was obviously in error because the responses on the heterologous series were too low. Therefore, comparisons were also made graphically. Thus, as shown later in figure 1 *A*, the plot of response *versus* log concentration of nitrogen shows that BAI regular tuberculin has some specificity; that is, for a given response, a higher concentration is required on the heterologous series than on the homologous. To be more specific than the control, a fraction must yield curves on the graph even farther apart than those for the control. For numerical comparisons to be valid, the homologous and heterologous curves should be parallel within the limits of experimental error. This obviously would be impossible if the fraction were specific because the heterologous curve would then coincide with the abscissa. Therefore, the more specific materials can be tested at concentrations as high as possible on the heterologous series and then, if the reactions are not sufficiently high, the calculated specificity will not be numerically correct, but the graphic demonstration of enhanced specificity will be none the less valid.

OBSERVATIONS

Material

The tuberculin used in this work was furnished by the Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture.

Human-type tubercle bacilli (Strains Pn, C, and Dt) were grown on a synthetic medium containing dextrose and glycerol as described by Dorset and Henley (2). Strain 110 of bovine-type bacilli was grown on a medium which has a lower asparagine and glycerol content than that just mentioned. At the age of eight to twelve weeks the cultures were heated in flowing steam for an hour and allowed to cool. Filtration through a fine wire screen and then through paper yielded the dilute culture filtrate.

Comparative bovine skin tests showed that unheated culture filtrate, sterilized by filtration through sintered glass, was neither significantly more potent nor more specific than material which had been heat-sterilized either at the normal pH of the filtrate (approximately 8.5) or after the pH had been adjusted to 7.0. This experiment showed that the heat-sterilization procedure used in the preparation of tuberculin apparently had no deleterious effect on the biological activity of the product.

Incidentally, another experiment showed that international standard tuberculin, prepared by Seibert,[2] was neither significantly more potent nor more specific than BAI regular tuberculin.

Fractionation of Human Tubercle Bacilli Culture Filtrate with Hydrochloric Acid

Fractionation of tubercle bacilli culture filtrate at its normal pH (approximately 8.5) by the addition of alcohol gave a series of fractions, none of which was significantly more specific than BAI regular tuberculin. Therefore, similar fractionations were carried out at pH levels below the neutral point.

> Three lots of culture filtrate, previously concentrated by ultrafiltration using Berkfeld candles covered with synthetic sausage casing, were adjusted while being stirred at room temperature to pH levels 5.0, 4.0, and 3.0 by the addition of N hydrochloric acid. The precipitates were removed by centrifugation (2,500 G.), and the supernatant solutions were made to 30 per cent alcohol. After removal of the resulting precipitates, additional solid fractions were obtained at 60 and 90 per cent alcohol.

Bovine skin tests of solutions of the fractions showed that the precipitates obtained on acidification were more specific than the control, while the fractions precipitated by alcohol were less potent and less specific.

That acidification, without the addition of any alcohol, precipitated the more specific material from culture filtrate was again shown as follows:

> Two lots of culture filtrate, previously concentrated in a hood draft and filtered, were made to pH 5.0 and 4.0, respectively. A second fraction was then obtained from each lot by adding alcohol to the supernatant solutions up to 90 per cent by volume. The results of this experiment, summarized in table 1, show that the more potent and more specific material was precipitated at pH 5.0 and that a greater amount was precipitated at pH 4.0. The materials precipitated by alcohol at both pH levels from the supernatant solutions were low in potency and in specificity.

Fractionation of unconcentrated culture filtrate from the batch of culture filtrate referred to in the preceding paragraph was effected at pH levels 5.0, 4.0, and 3.0, after which the supernatant solution was made to 90 per cent alcohol. The yields of the four fractions, based on the nitrogen in the culture filtrate, were 11, 8, 0.5, and 7 per cent, respectively. Again the more specific material was precipitated by the acid, while the material precipitated by the alcohol was low in both potency and specificity.

Although most of the homologous activity was in the precipitate obtained at pH 5.0 from either dilute or concentrated culture filtrate, the process was unwieldy because the precipitate did not settle; large volumes had to be centrifuged. To avoid this difficulty, precipitation was effected at a pH 3.8 and the solid was then redissolved at pH 8.0 and fractionated from smaller volumes. The supernatant solution thus obtained was neutralized and concentrated. Bovine

[2] A sample of international standard tuberculin was kindly furnished by the Microbiological Institute of the Public Health Service, Bethesda, Maryland.

skin tests gave the homologous potency and specificity of the material in the supernatant solution as 0.1 and 0.2, respectively. This result was confirmed in several experiments to assure that the discarded supernatant solution was low in potency and specificity. The solid precipitated at pH 3.8 from dilute culture filtrate was reprecipitated six times at pH 3.8 to remove as much color as possible. In the last reprecipitation the supernatant solution was light yellow. The yield of solid was approximately 1.25 gm. per liter of culture filtrate, of which 5.5 per cent was ash and 16 per cent was nitrogen (ash-water free).

One method of fractionating the material precipitated at pH 3.8 was to divide it into two parts at an intermediate pH level of precipitation and then to divide each of these parts into two further fractions.

TABLE 1

FRACTIONATION OF CONCENTRATED TUBERCLE BACILLI CULTURE FILTRATE* WITH HYDROCHLORIC ACID AND ALCOHOL

FRACTION	YIELD OF SOLID	ASH	NITROGEN†	YIELD‡	HOMOLOGOUS POTENCY§	SPECIFICITY‖
	gm.	*per cent*	*per cent*	*per cent*		
Precipitated at pH 5.0	3.92	8.53	13.29	7	2.5	2.3
Precipitated from supernatant solution at 90 per cent alcohol concentration	33.36	22.92	8.35	31	0.5	0.6
Precipitated at pH 4.0	6.22	6.35	13.69	12	1.4	1.1
Precipitated from supernatant solution at 90 per cent alcohol concentration	30.08	23.12	7.55	26	0.4	0.6

* Each batch consisted of 7 liters of culture filtrate concentrated tenfold.

† Ash- and water-free basis.

‡ Yield is based on the nitrogen in the original culture filtrate.

§ The potency values were calculated on a nitrogen basis with Bureau of Animal Industry regular tuberculin taken as unit potency.

‖ The specificity is the ratio of homologous to heterologous potency.

Thus, a solution of the material was adjusted to pH 5.0 to precipitate *fraction 1*. Adjustment of the supernatant solution to pH 4.5 precipitated *fraction 2*. Each fraction was then dissolved. The solution of *fraction 1* was adjusted to pH 5.1 to precipitate *fraction 3*, and adjustment of the supernatant solution to pH 4.7 precipitated *fraction 4*. Similarly, *fraction 2* was divided into *fractions 5* and *6* by precipitating from the solution of *fraction 2* at pH 4.82. The amino acid analysis of *fraction 5* is given in a later section. Bovine skin tests showed that most of the more specific material was in the middle fractions, *4* and *5*, but that none of the fractions was very much more specific than the others.

In a second fractionation, the material precipitated from dilute culture filtrate at pH 3.8 was dissolved at pH 8 and precipitated at pH 4.0. The resulting precipitate was then recovered and redissolved. Reprecipitations were effected at 0.1 pH unit intervals from pH 4.2 to 5.0. Samples of the precipitate and supernatant solution at each stage were removed for testing. The results showed that the material could be reprecipitated almost quantitatively at pH levels up to and including 4.7 and that the material in the supernatant solutions thus

obtained was low in potency. At pH 4.8 most of the activity was still in the precipitate, but fairly specific material was in the supernatant solution. At pH 5.0, most of the activity was in the supernatant solution.

Fractionation beyond removal of material soluble at pH 4.7 and material insoluble at pH 5.2 did not give reproducible results, however, because small differences in the procedure yielded fractions which differed somewhat in specificity. Furthermore, excessive amounts of active material had to be sacrificed in the preparation of more specific materials. Therefore, fractionation with acid was used only to concentrate and to purify the protein to some extent. The resulting material was then further fractionated by another method as described in a later section.

Fractionation of Bovine Tubercle Bacilli Culture Filtrate

Tuberculin for intradermic testing is often produced from human-type tubercle bacilli cultures because this type grows well and the product gives a positive test for both human and bovine tuberculosis. However, for the preparation of a material which would be more specific for bovine tuberculosis, it would seem more logical to fractionate the product of the bovine-type bacilli. Therefore, the following fractionations were carried out.

Bovine tubercle bacilli[3] culture filtrate, at 0° C., was adjusted to pH 4.95 by the slow addition of concentrated hydrochloric acid during stirring. In contrast to the results obtained at this pH with human-type tubercle bacilli culture filtrate, the precipitate settled, leaving a clear supernatant solution. As the supernatant solution was further acidified, no precipitate appeared until the pH was below 4.4. A second precipitate was taken at pH 3.5. An aliquot of the supernatant solution diluted with alcohol to 90 per cent yielded a third solid. Tests of these fractions, summarized in table 2, show that most of the active material was precipitated at pH 4.95 and pH 3.5. The large fraction precipitated by adding alcohol to the supernatant solution to 90 per cent was neither potent nor specific. The amino acid analysis of the fraction precipitated at pH 4.95 is given in a later section.

In a second fractionation of bovine-type tubercle bacilli culture filtrate, the total precipitate was taken at pH 3.2 and redissolved (*fraction B 2*). A sample of the supernatant solution was neutralized and then concentrated threefold in a vacuum desiccator (*fraction B 1*). The solution of *fraction B 2* was adjusted to pH 5.04 and supercentrifuged to separate the solid (*fraction B 3*). The supernatant solution was adjusted to pH 4.32 to precipitate *fraction B 4*. The solution of *fraction B 4* was adjusted to pH 4.72 to precipitate *fraction B 5*. The accompanying supernatant solution obtained at pH 4.72 was adjusted to pH 4.32 to yield a supernatant solution (*fraction B 6*) and a precipitate (*fraction B 7*).

Bovine skin tests of these fractions, summarized in table 3, show that the initial supernatant solution was low in both potency and specificity. Fractionation of the material precipitated at pH 3.2 yielded some fractions which were fairly potent and specific.

[3] This strain, No. 110, was obtained from the Division of Pathology, Ministry of Agriculture, Dominion of Canada.

Fraction B 7 was further tested on fifty sensitized bovines divided into five equal groups. Each group was sensitized to one of the following: *M. tuberculosis* var. *hominis; M. tuberculosis* var. *bovis; M. paratuberculosis; M. tuberculosis* var. *avium*; and *M. phlei*. The potency values for the various sensitizations were: *hominis* sensitization, 2.3; *bovis* sensitization, 7.6; *paratuberculosis* sensitization, 1.4; *avium* sensitization, 1.6; and *phlei* sensitization, 1.3. The specificity for bovine tuberculosis sensitization over paratuberculosis was 5.4.

TABLE 2

FRACTIONATION OF 10 LITERS OF BOVINE-TYPE TUBERCLE BACILLI CULTURE FILTRATE

FRACTION	YIELD OF SOLID	YIELD*	HOMOLOGOUS POTENCY	SPECIFICITY
	gm.	*per cent*		
1. Precipitated at pH 4.95	6.10	3.9	10.0	6.2
2. Precipitated at pH 3.5	3.13	2.7	2.0	3.9
3. Precipitated from supernatant solution at 90 per cent alcohol concentration	21.0	5.4	0.63	0.60

* Yield is based on the nitrogen in the original culture filtrate.

TABLE 3

FRACTIONATION OF 19.5 LITERS OF BOVINE TUBERCLE BACILLI CULTURE FILTRATE

FRACTION	YIELD*	HOMOLOGOUS POTENCY	SPECIFICITY
	per cent		
B 1. Supernatant solution at pH 3.2 (neutralized and concentrated threefold)	93.0	0.07	0.6
B 2. Precipitated at pH 3.2 (redissolved)	4.3	3.4	2.6
B 3. Precipitated at pH 5.04 from solution of B 2	0.33	6.2	2.5
B 4. Precipitated from supernatant obtained at pH 4.32	0.80	0.8	3.0
B 5. Precipitated at pH 4.72	0.65	5.1	6.2
B 6. Supernatant at pH 4.32	0.09	2.0	0.9
B 7. Precipitated at pH 4.32	1.9	6.0	6.3

* Yield is based on the nitrogen in the original culture filtrate.

The foregoing fractionations and tests showed that bovine tuberculin could be fractionated satisfactorily, but the results did not indicate that a specific material could be more readily obtained from the culture filtrate of the bovine-type bacilli than from that of the human type.

Fractionation of Tuberculin from Phenol Solution

Preliminary trials showed that solid tuberculin fractions could be dissolved in phenol and fractionated by the addition of alcohol to yield materials which were more specific than BAI regular tuberculin. A typical fractionation was as follows:

Eighteen grams of an acid-precipitated fraction from human tubercle bacilli culture filtrate was sprinkled into 600 gm. of 90 per cent phenol which was being stirred. Slow addition of alcohol to the thick, dark brown solution up to 10 per

cent by weight gave practically no precipitate. Additional alcohol to make the concentration 20 per cent by weight yielded a rubbery, black solid, not readily soluble in water. More of the rubbery precipitate was obtained when the supernatant solution was made up to 25 per cent alcohol. Additional fractions precipitated at 30, 35, 43, and 60 per cent alcohol by weight were light tan and readily soluble in water. A final fraction was precipitated when the 60 per cent alcohol supernatant solution was evaporated to half its original volume and then diluted with an equal volume of ether. The numerical values of the specificities of these fractions are not given because the heterologous responses were too low. However, comparisons made graphically showed that, as the alcohol concentration was increased up to 35 per cent, the heterologous potency of the precipitated material became lower. Subsequent fractions showed somewhat higher and increasing heterologous potency. Concerning homologous potency, there was not much difference among the fractions, except that the material precipitated at 35 per cent alcohol concentration was the most potent.

Since the foregoing fractionation, like the acid fractionation, yielded only small fractions of the highest specificity, it seemed desirable to utilize this procedure only to the extent of removing the black material precipitated at 25 per cent alcohol concentration and the more soluble material which was precipitated by ether from the concentrated 60 per cent alcohol supernatant solution.

Combined Procedure

The combined procedure devised for the preparation of the most specific material was as follows:

Dilute culture filtrate, pH 9.2, containing 0.71 mg. of nitrogen per ml., was adjusted during stirring at 0° C. to pH 3.8 by the dropwise addition of concentrated hydrochloric acid. The resulting precipitate was dissolved at pH 8 and reprecipitated with normal hydrochloric acid at pH 4.2, 4.4, and 4.6, successively. The solution of the final precipitate was adjusted to pH 5.2 and supercentrifuged, after which the supernatant solution was adjusted to pH 4.6, and the resulting precipitate was recovered and dissolved at pH 8. The pH 8 solution was dialyzed for thirty-six hours against three one-liter portions of distilled water. The dialysate was concentrated for testing. A solid was precipitated from the "endo" solution (that remaining within the membrane) at pH 4.5 and dissolved. To this solution, alcohol was added to 90 per cent by weight to precipitate a solid, which was washed with absolute alcohol, then with ether, and dried. The yield of dry material was 83 mg. per 100 ml. of original culture. Fourteen grams of the solid were sprinkled into 500 gm. of 90 per cent phenol with stirring. The phenol solution was diluted with alcohol to 25 per cent by weight to precipitate 6.9 gm. of black solid. The supernatant solution was adjusted to 60 per cent alcohol by weight to precipitate 5.44 gm. of solid. The supernatant solution (60 per cent alcohol) was evaporated in a hood draft to half of its original volume and diluted with three times its volume of ether to obtain 0.8 gm. of solid.

The results of bovine skin tests of the fractions, graphed in figure 1, show the following: Acid fractionation yielded a material which was more specific than

BAI regular tuberculin. The dialysate obtained in the fractionation was low in potency and was much less specific than regular tuberculin. Precipitation of the acid fraction from phenol by the addition of alcohol yielded the most specific material at 60 per cent alcohol; 6 per cent yield based on the nitrogen present in the original culture filtrate. Therefore, the three-step process consisting of acid fractionation, dialysis, and fractionation of the protein from phenol yielded a material which was more specific by high significance than BAI regular tuberculin. These results were confirmed in additional experiments.

TABLE 4

AMINO ACID CONTENT OF TWO TUBERCULIN FRACTIONS

(Expressed as Per Cent of Total Nitrogen in the Form of the Amino Acid)

AMINO ACID	HUMAN TUBERCULIN FRACTION*	BOVINE TUBERCULIN FRACTION†
	per cent	*per cent*
Alanine	13.2	9.2
Asparatic acid	7.9	8.0
Arginine	15.9	15.6
Cystine	0.2	0.3
Glutamic acid	8.0	8.7
Glycine	7.7	8.1
Histidine	3.5	4.1
Isoleucine	4.6	4.3
Leucine	6.3	6.3
Lysine	6.0	4.1
Methionine	1.2	1.3
Phenylalanine	2.1	2.2
Proline	4.1	4.2
Serine	4.3	3.1
Threonine	4.3	5.4
Tryptophan	1.4	1.7
Tyrosine	1.8	1.6
Valine	2.7	5.4
Total	95.2	93.6

* Nitrogen, 16.4 per cent.

† Nitrogen, 13.7 per cent.

Amino Acid Content of Two Tuberculin Fractions

During the course of the work on the fractionation of tuberculin, two fractions were analyzed for amino acids. Although the fractions chosen were not the most potent and specific, they were the best available at the time the analyses were initiated. The results are presented here mainly because of the paucity of such data for tuberculin in the literature; only two papers on this subject were found (14, 15).

The human tuberculin fraction analyzed was *fraction 5* referred to in the sixth paragraph under *Fractionation with Hydrochloric Acid* (above). The solid precipitated from tubercle bacilli culture filtrate at pH 3.8 had been dissolved and the solution fractionated at pH 5.0. The precipitate obtained at pH 5.0 had

been redissolved and the solution adjusted to pH 5.1. After removal of the precipitate, the supernatant solution had been adjusted to pH 4.7 to precipitate the fraction analyzed.

The bovine tuberculin fraction analyzed was the one precipitated in the first experiment on the fractionation of bovine-type culture filtrate upon adjusting the culture filtrate to pH 4.95 (fraction 1, table 2).

Amino acids were determined microbiologically except that tryptophan was determined colorimetrically.[4] The procedure is outlined by Spies and co-workers (16). The results are given in table 4.

DISCUSSION

On the assumption that the tuberculin skin test involves an allergen-antibody reaction in which the allergen is a protein produced by the tubercle bacilli, alteration of the specificity of the tuberculin may be attempted in two ways. In the first, it may be assumed that certain functional groups of the protein react with the various species of antibodies. Thus, a certain group may give the homologous reaction while other groups give heterologous reactions. On this hypothesis, alteration of specificity may be attempted by physical or chemical treatment in the hope of quenching the heterologous activity accompanied by little or no impairment of the homologous activity. Experiments based on this idea have been reported by Green (17) in England and also by Baisden and co-workers in this country (9). Although the results of such treatments have so far not been entirely successful, the large number of possible treatments make it premature to reject the hypothesis on which such experiments are based.

A second hypothesis upon which attempts at alteration of specificity may be based is that there may be several proteins in tuberculin, each of which has a certain potency with respect to the various sensitizations. Thus, one protein may have a high reactivity on a tuberculin sensitization, a medium potency on a johnin sensitization, and practically no reactivity on, say, a phlein sensitization, while another may have very low reactivity on all sensitizations except tuberculin. Hence, on the basis of this hypothesis, favorable alteration of specificity may be attempted by fractionating the tuberculin proteins.

The work herein reported was based on the second hypothesis. Several fractionation procedures have been combined in order to prepare a material of high tuberculin potency and relatively low heterologous potency. Thus, precipitation at various pH levels was used to prepare fractions of fairly high specificity but, when this procedure was extended to the separation of many fractions, the results became somewhat erratic in terms of the specificities of the fractions and the yields diminished to the vanishing point. Therefore, fractionation with acid was utilized only to separate and partially purify the active material. Dialysis was then utilized to remove much nonspecific material. Final fractionation was effected by precipitation of the most specific material from phenol solution by the addition of alcohol. The combined procedure consists of three steps, each of which contributes something to the purification of the protein.

[4] These analyses were carried out by Mrs. D. C. Chambers, Bureau of Agriculture and Industrial Chemistry, Allergy Division.

Field tests on herds having Johne's disease but free of tuberculosis are now necessary to determine whether the resulting fraction is sufficiently specific for practical use.

SUMMARY

On the basis of bovine skin tests carried out on animals sensitized to heat-killed tubercle bacilli (*Mycobacterium tuberculosis*) and to heat-killed Johne's bacilli (*M. paratuberculosis*), certain fractions prepared from tubercle bacilli culture filtrate were more specific than Bureau of Animal Industry (BAI) regular tuberculin. The most specific fraction was prepared by a three-step process. (*1*) The culture filtrate was fractionated by acidification to remove material soluble at pH 4.7 and insoluble at pH 5.2. (*2*) Dialysis of the acid-precipitated fraction thus prepared left most of the active material in the "endo" solution; the dialysate contained nonspecific material. (*3*) The "endo" solution was treated with alcohol, and the precipitate thus formed was dissolved in 90 per cent phenol and fractionated by the addition of alcohol.

At 60 per cent alcohol a fraction was obtained which was even more specific than the acid-precipitated fraction. This fraction, amounting to 6 per cent based on the nitrogen in the original culture filtrate, was more specific to a high degree of significance than was the BAI regular tuberculin.

Amino acid compositions were determined of two fractions—one isolated from human-type tubercle bacilli culture filtrate and the other from bovine-type tubercle bacilli culture filtrate.

SUMARIO

Estudios de la Fraccionación de la Tuberculina y la Johnina: I. Fraccionación de las Proteínas de la Tuberculina

De acuerdo con las cutirreacciones bovinas ejecutadas en animales sensibilizados a bacilos tuberculosos (*Mycobacterium tuberculosis*) matados al calor y a bacilos de Johne (*M. paratuberculosis*) matados al calor, ciertas fracciones preparadas de filtrados de cultivos de bacilos tuberculosos fueron más específicas que la tuberculina regular de la Oficina de Industrial Animal de los E. U. A. La fracción más específica fué preparada con un procedimiento de tres tiempos: (1) Se fraccionó el filtrado del cultivo por acidificación para eliminar las materias solubles a un pH de 4.7 e insolubles a un pH de 5.2. (2) La diálisis de la fracción precipitada con ácido y preparada en dicha forma dejó la mayor parte de las materias activas en la solución de endo; el dializado contenía materias anespecíficas. (3) Se trató la solución de endo con alcohol y el precipitado así formado fué disuelto en 90 por ciento de fenol y fraccionado por la adición de alcohol. Con 60 por ciento de alcohol, se obtuvo una fracción aun más específica que la precipitada con ácido. Esa fracción, que representaba 6 por ciento a base del nitrógeno del filtrado del cultivo primitivo, fué más específica, en grado significativo, que la tuberculina regular de la Oficina de Industria Animal.

Se determinó la composición en amino-ácidos de dos fracciones: una aislada del filtrado del cultivo de bacilos tuberculosos de tipo humano y la otra del filtrado del cultivo de bacilos tuberculosos de tipo bovino.

RESUME

Etudes sur le fractionnement de la tuberculine et de la johnine: I. Fractionnement des protéines de la tuberculine

En accord avec les tests cutanés bovins effectués sur des animaux sensibilisés au bacille tuberculeux tué par la chaleur (*Mycobactérium tuberculosis*) et au bacille de johne tué par la

chaleur (*M. paratuberculosis*), certaines fractions préparées à partir du filtrat de cultures de bacilles tuberculeux étaient plus spécifiques que la tuberculine ordinaire du *Bureau of Animal Industry*. La fraction la plus spécifique était préparée par un procédé en trois temps. (1) Le filtrat de culture était fractionné par acidification pour extraire la matière soluble à pH 4,7 et insoluble à pH 5,2; (2) la dialyse de la fraction précipitée par l'acide ainsi préparée laissait la plus grande partie de la substance active dans l'endo-solution; le dialysat contenait la substance non-spécifique; (3) l'endo-solution était traitée par l'alcool et le précipité ainsi formé était dissous dans le phénol à 90% et fractionné par addition d'alcool. Lorsque le taux d'alcool était de 60%, on obtenait une fraction qui était encore plus spécifique que la fraction précipitée par l'acide. Cette fraction qui représentait 6%, en prenant pour base l'azote contenu dans le filtrat de la culture initiale, atteignait un degré de spécificité considérablement plus élevé que la tuberculine ordinaire du *Bureau of Animal Industry*.

L'aminoacide a été déterminé dans deux des fractions, l'une isolée d'un filtrat de culture de bacilles tuberculeux, type humain, et l'autre d'un filtrat de cultures de bacilles tuberculeux, type bovin.

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STUDIES ON THE FRACTIONATION OF TUBERCULIN AND JOHNIN[1]

II. Extraction of Tuberculin Proteins with Phenol

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INTRODUCTION

Experiments were described in the first paper of this series (1) which culminated in a three-step process for the preparation of a tuberculin fraction which was more specific according to skin tests on artificially sensitized cows than the intrademic tuberculin (BAI regular tuberculin) used in official testing by the Bureau of Animal Industry. The present paper concerns extraction of aqueous solutions of tuberculin proteins with phenol. The materials and the methods of testing fractions are presented in the previous report (1).

OBSERVATIONS

The materials obtained by phenol extraction and subsequent fractionation were compared for potencies with regular BAI tuberculin in skin tests made on 10 cattle sensitized to *Mycobacterium tuberculosis* var. *bovis* and on 10 cattle sensitized to *Mycobacterium paratuberculosis*. The responses as measured by increases in skin thickness resulting from the injection of four concentrations of each material were related to the log of the nitrogen contents of the materials so that the nitrogen content could be estimated, which would give a selected response. From these estimations the comparative potencies of unknowns and the regular tuberculin could be obtained on the two sensitizations.

Countercurrent Extraction

The fact that the active material precipitated from tubercle bacilli culture filtrate by hydrochloric acid was soluble in phenol as well as water suggested the possibility of fractionation by means of countercurrent extraction between water and phenol. The main difficulty in such a procedure was the formation of stable emulsions; the protein apparently acted as an emulsifying agent. However, centrifugation for two or more hours at approximately 5° C. separated the layers, which became clear on standing for a few minutes at room temperature.

To prepare a sample for countercurrent extraction, tubercle bacilli culture filtrate was adjusted during stirring at 0° C. to pH 3.8 by the slow addition of concentrated hydrochloric acid. The next day the precipitate was recovered, dissolved at pH 8.0, and reprecipitated at pH levels 4.0, 4.4, and 4.7. The resulting

[1] From the Allergen Research Division, Bureau of Agricultural and Industrial Chemistry, and the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington 25, D. C.; and the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, U. S. Department of Agriculture, Auburn, Alabama.

solid was dissolved at pH 8.0 and the solution was supercentrifuged at pH 5.2. The supernatant solution was neutralized and then dialyzed for forty-eight hours against several changes of distilled water, after which the "endo" solution (that remaining inside the membrane) contained 9.8 per cent of the nitrogen present in the original culture filtrate.

A 100-ml. sample of the "endo" solution was cooled to 5° C. and then shaken at intervals of 0.5 hour with 100 ml. of cold 90 per cent phenol in tube 0, aqueous end, after which the layers were separated by centrifugation for two hours at 5°C. After the tube had stood for a few minutes, the clear, dark brown lower phenol layer was moved to tube 1. Fresh 90 per cent phenol was added to the aqueous layer remaining in tube 0 and fresh water was added to the phenol layer in tube 1. After mixing thoroughly, the mixtures were centrifuged and the lower layers moved again. Countercurrent extraction was thus effected with four tubes. The lower layer of tube 0 and the upper layers in tubes 2 and 3 were colorless. Each of the eight layers was separated, frozen, and stirred with nine volumes of absolute alcohol and a few drops of normal hydrochloric acid. Significant quantities of precipitate were obtained only from the upper layer of tube 0

TABLE 1

COUNTERCURRENT FRACTIONATION OF AN ACID PRECIPITATED FRACTION OF TUBERCULIN

FRACTION	YIELD OF SOLID	NITROGEN CONTENT OF SOLID	YIELD, NITROGEN BASIS	HOMOLOGOUS POTENCY*	SPECIFICITY*
	mg.	*per cent*	*per cent*		
Aqueous end	350	12.8	7.1	0.55	0.26
Phenol end	700	14.6	16.0	4.2	4.4

* The homologous potency was calculated on a nitrogen basis from tests on sensitized cows with BAI regular tuberculin taken as unit potency and specificity. The specificity is the ratio of the homologous to the heterologous potency.

(aqueous end) and from the lower layer of tube 3 (phenol end). Therefore, the extraction separated two rather distinct materials, one of which remained in the aqueous layer even after several extractions with phenol, while the other remained in the phenol layer even after several extractions with water.

Yields and results of skin tests with the two main fractions on sensitized cattle are summarized in table 1. The data show that the extraction separated a nonspecific material in the aqueous end from a fairly specific material in the phenol end.

Extraction of Tuberculin with Phenol

Although countercurrent extraction separated a nonspecific from a fairly specific material, the yields based on the nitrogen in the original culture filtrate were low because an acid-precipitated fraction was prepared before the countercurrent fractionation was begun. Since the phenol-extracted material was the more specific, it seemed advisable to extract culture filtrate with phenol directly.

For this purpose, 35 liters of culture filtrate were evaporated to 3.5 liters in a vacuum evaporator and then dialyzed for two days against running tap water.

The "endo" solution was concentrated to 3.5 liters again, passed through a Sharples continuous supercentrifuge twice, and filtered through a K 5 pad in a Seitz filter. The filtrate contained 39 per cent of the nitrogen in the original culture filtrate.

A 2-liter sample of the filtered solution (fraction 0) was extracted successively with three portions of phenol; 500, 300, and 200 ml. measured as 90 per cent phenol, the layers being separated by centrifugation. The aqueous residue was washed with ether, concentrated in a vacuum still, and diluted with alcohol to 90 per cent concentration by weight, whereupon 31.52 gm. of solid were obtained (*fraction 1*). The yield was 17 per cent, based on the nitrogen in the concentrated "endo" solution.

The combined phenol extract was frozen and stirred with nine volumes of absolute alcohol and a few drops of normal hydrochloric acid. The resulting precipitate, after being washed with alcohol and with ether and dried, weighed 22.2 gm., the yield being 32 per cent, based on the nitrogen in the concentrated "endo" solution. The solid was dissolved in water and reprecipitated at pH 4.0. It was redissolved and the solution was supercentrifuged at pH 5.4. The precipitate was dissolved for test (*fraction 2*). The supernatant solution was adjusted to pH 7.0, yield 8 per cent based on the nitrogen in the original culture filtrate (*fraction 3*).

TABLE 2

HOMOLOGOUS POTENCIES AND SPECIFICITIES OF FRACTIONS PREPARED FROM TUBERCULIN BY EXTRACTION WITH PHENOL

FRACTION	HOMOLOGOUS POTENCY	SPECIFICITY
0. Concentration "endo" solution	2.1	0.64
1. Aqueous residue after extraction with phenol	2.4	0.11
2. Material removed from phenol extract on fractionation with hydrochloric acid	0.36	1.1
3. Phenol extract after fractionation with hydrochloric acid	3.1	6.5

The results of the tests, given in table 2, show that extraction of aqueous tuberculin proteins with phenol left a nonspecific material in the aqueous residue (*fraction 1*). Furthermore, fractionation of the material in the phenol extract by precipitation with acid removed some material which was neither potent nor specific (*fraction 2*). The main fraction (*fraction 3*) obtained from the phenol extract was more potent and more specific, to a high degree of significance, than BAI regular tuberculin. That phenol extracted the more specific material from an aqueous solution of tuberculin proteins was confirmed in experiments involving phenol extraction of dilute and of concentrated tubercle bacilli culture filtrate, as well as concentrated "endo" solution of tuberculin proteins.

DISCUSSION

In this and a previous paper, four methods which are useful for the fractionation of tuberculin proteins are described. Each of these methods yields some

fractions that are more specific than BAI regular tuberculin. The methods are: (*1*) fractional precipitation at various pH levels by addition of hydrochloric acid; (*2*) fractional precipitation by the addition of alcohol to a phenol solution of tuberculin proteins; (*3*) dialysis to remove small molecules; and (*4*) phenol extraction of the active material from aqueous solution of tuberculin proteins.

Of these four methods, the first two differ from the second two in that the former are fractional precipitations. In this type of fractionation the fractions obtained have similar properties, differing from the preceding or subsequent ones only in degree. The fractionation is not necessarily sharp, and usually the successive fractions exhibit a gradation in properties from one to the other. These methods yield some fractions which are significantly more specific than the control but none which is significantly less specific.

On the other hand, the latter two methods, dialysis and extraction with phenol, gave a much sharper fractionation in that the fractions obtained differed markedly in properties. Each of these methods gave two fractions, one of which was more specific than BAI regular tuberculin while the other was less specific. Obviously, the latter procedures are the more efficient, but the former are also useful for the removal of a certain amount of material which is not very specific. The combination of the four procedures has been used in order to prepare material in reasonable yields for field tests.

SUMMARY

Countercurrent fractionation between water and phenol of an acid-precipitated fraction prepared from tubercle bacilli culture filtrate yielded a nonspecific material in the aqueous end of the system and a fairly specific protein in the phenol end. Specificity was determined by skin tests on cattle sensitized with *M. tuberculosis* var. *bovis* or with related acid-fast bacilli.

Tubercle bacilli culture filtrate, which had been concentrated and dialyzed, was extracted with phenol. The active material was precipitated from the phenol extract by alcohol and then dissolved in water and fractionally precipitated by hydrochloric acid. The fraction which was insoluble at pH 4.0 and soluble at pH 5.4 was more potent and more specific, to a high degree of significance, than Bureau of Animal Industry regular tuberculin. The yield was 8 per cent, based on the nitrogen in the original culture filtrate.

SUMARIO

Estudios de la Fraccionación de la Tuberculina y la Johnina: II. Extracción con Fenol de las Proteínas de la Tuberculina

La fraccionación contra-corriente entre el agua y el fenol de una fracción precipitada con ácido de un filtrado de cultivo de bacilos tuberculosos dió una substancia anespecífica en el extremo acuoso de la combinación y una proteína bastante específica en el extremo fenólico. La especificidad fué determinada por medio de cutirreacciones en bovinos sensibilizados con *M. tuberculosis* var. *bovis* o con bacilos ácidorresistentes afines.

El filtrado de cultivo de bacilos tuberculosos, que había sido concentrado y dializado, fué extraído con fenol. Las materias activas fueron precipitadas con alcohol del extracto fenólico y luego disueltas en agua y precipitadas fraccionadamente con ácido chorhídrico. La frac-

ción insoluble a un pH de 4.0 y soluble a un pH de 5.4, fué más potente y más específica, en forma muy significativa, que la tuberculina regular de la Oficina de Industria Animal de los E. U. A. El rendimiento representó 8 por ciento a base del nitrógeno contenido en el primitivo filtrado del cultivo.

RESUME

Etudes sur le fractionnement de la tuberculine et de la johnine: II. Extraction des protéines de la tuberculine par le phénol

Inversement, le fractionnement entre l'eau et le phénol d'une fraction obtenue par précipitation acide préparée à partir d'un filtrat de culture de bacilles tuberculeux a donné une substance non-spécifique à la fin de la phase aqueuse du système et une protéine de spécifité moyenne à la fin de la phase phénol. La spécifité était déterminée par des tests cutanés sur des bovins sensibilisés au *M. tuberculosis* var. *bovis* ou avec des bacilles apparentés acido-résistants.

Le filtrat de cultures de bacilles tuberculeux était extrait par le phénol après concentration et dialyse. La substance active était précipitée par l'alcool à partir de l'extrait phénolique, puis dissoute dans l'eau et précipitée, par fractions avec l'acide chlorydrique. La fraction insoluble au pH 4 et soluble au pH 5, 4 avait un degré d'activité et de spécificité considérablement plus marqué que la tuberculine régulière du *Bureau of Animal Industry*. Le rendement était de 8%, en prenant comme base l'azote du filtrat de la culture originale.

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STUDIES ON THE FRACTIONATION OF TUBERCULIN AND JOHNIN[1]

III. Fractionation of Johnin Proteins

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INTRODUCTION

Procedures similar to those reported in the first two papers of this series (1, 2) for the preparation of fairly specific tuberculin fractions have been applied to Johne's bacilli culture filtrate. This was done in order to concentrate the active material and then fractionate from the concentrate a material more specific than the intradermic-strength johnin (BAI regular johnin) now used by the Bureau of Animal Industry. However, some of the methods used with tuberculin proteins were not applicable to johnin proteins because most of the active material in the latter is not precipitated on addition of hydrochloric acid. Various other procedures were tried, such as precipitation with acid and alcohol, phosphotungstic acid, and picric acid, but the results were unsatisfactory because the material precipitated was obtained in small yields and was not more specific than BAI regular johnin. More successful results are reported in the present paper. The method of testing fractions is given in the first paper of this series (1). In brief, it consisted of making comparative skin tests of fractionated johnins and regular BAI johnin, each at four levels of nitrogen concentration, on 10 cattle sensitized to *Mycobacterium paratuberculosis* and on 10 cattle sensitized to *Mycobacterium tuberculosis* var. *bovis*. The responses were related to the logs of the nitrogen concentration and the comparative potencies of an unknown and the regular johnin on each of the two sensitizations estimated from the concentrations required to produce a given response.

OBSERVATIONS

Fractionation Procedures

Precipitation from solution in phenol: Johne's bacilli culture filtrate was concentrated fivefold in a vacuum desiccator and made to 90 per cent alcohol. The precipitate thus formed was recovered by centrifugation, washed with alcohol and ether, and dried. The solid, which contained 6 per cent nitrogen and represented 38 per cent of the nitrogen in the original culture filtrate, was ground and then sprinkled into 100 gm. of 90 per cent phenol which was being stirred. After being stirred for two hours, the phenol solution was centrifuged, and the material insoluble in phenol was recovered and dissolved in water.

[1] From the Allergen Research Division, Bureau of Agricultural and Industrial Chemistry, and the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington 25, D. C.; and the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, U. S. Department of Agriculture, Auburn, Alabama.

The phenol solution was fractionated by the addition of alcohol and ether to obtain three precipitates: (*1*) precipitated at 30 per cent alcohol by weight; (*2*) precipitated after further addition of alcohol to 60 per cent by weight; and (*3*) precipitated after dilution of the supernatant 60 per cent alcohol solution with one volume of ether. The yields and results of tests on sensitized cows are given in table 1.

These data show that the material precipitated on making the concentrated filtrate to 90 per cent alcohol was fractionated to yield first a fairly potent but not particularly specific material which was insoluble in phenol. The phenol-soluble part was then separated into three fractions which were less potent but more specific than BAI regular johnin. The fraction precipitated at 60 per cent alcohol concentration was the most specific.

TABLE 1

HOMOLOGOUS POTENCY AND SPECIFICITY OF SOME JOHNIN FRACTIONS PREPARED FROM THE SOLID PRECIPITATED FROM CONCENTRATED JOHNIN AT 90 PER CENT ALCOHOL

FRACTIONS OBTAINED FROM PHENOL SOLUTION	YIELD*	HOMOLOGOUS POTENCY†	SPECIFICITY†
	per cent		
Material insoluble in phenol	12	0.9	1.1
First precipitate (on adding alcohol to 30 per cent concentration)	15	0.4	2.3
Second precipitate (on increasing concentration of alcohol to 60 per cent)	5	0.6	4.2
Third precipitate (on adding equal volume of ether to 60 per cent alcohol solution)	6	0.4	3.6

* The yield is based on the nitrogen in original culture filtrate.

† The homologous potency and specificity are the values on a nitrogen basis compared with those for BAI regular johnin taken as unit potency and specificity.

Dialysis: Dialysis, previously found valuable for removing inactive material from tubercle bacilli culture filtrate, was also useful for the purification of Johne's bacilli culture filtrate. This was shown by concentrating 19 liters of Johne's bacilli culture filtrate in a hood draft to 1,440 ml. of thick solution and dialyzing the concentrate for a week against eight 2-liter portions of distilled water. Both the dialyzate and the "endo" solution (solution inside the membrane) were then concentrated for testing. The results, summarized in table 2, show that dialysis of concentrated johnin separated a fairly inactive material in the dialyzate from the more potent material in the "endo" solution.

Countercurrent extraction of johnin proteins: Since countercurrent extraction of tuberculin fractions separated a less specific from a more specific material (2), this procedure was applied to the fractionation of Johne's bacilli culture filtrate.

For this experiment 26 liters of Johne's bacilli culture filtrate were passed through a continuous supercentrifuge to remove bacilli and dross and then were concentrated in a vacuum evaporator to 3 liters. The concentrated solu-

tion was dialyzed for two hours against several changes of distilled water while the bag was moved at intervals to expedite dialysis. The resulting "endo" solution was then concentrated to 2.5 liters and further dialyzed for three days against several changes of distilled water. The "endo" solution was filtered through a K 7 pad and then through a sterilizing pad in a Seitz filter. The filtrate was evaporated to 1 liter and dialyzed overnight, after which time the "endo" solution contained 17 per cent of the nitrogen in the original culture filtrate.

A sample of the "endo" solution was fractionated by countercurrent extraction with phenol. Thus, in tube 0, the sample was extracted with phenol and the lower (phenol-rich) layer transferred to tube 1 which contained an equal volume of water. Fresh 90 per cent phenol was added to tube 0 and the contents of both tubes were stirred. After centrifugation, the lower layer of tube 1 was moved to tube 2 and that from tube 0 to tube 1, and fresh phenol was added to tube 0. This process was extended to nine tubes. The emulsion obtained on mixing the aqueous and phenol layers was resolved into two layers by centri-

TABLE 2

HOMOLOGOUS POTENCY AND SPECIFICITY OF THE DIALYSATE AND "ENDO" SOLUTION PREPARED BY DIALYSIS OF CONCENTRATED JOHNE'S BACILLI CULTURE FILTRATE

FRACTION	YIELD*	HOMOLOGOUS POTENCY	SPECIFICITY
	per cent		
"Endo" solution	34	13.0	1.0
Dialysate	66	0.5	0.2

* The yield is based on the nitrogen in the original culture filtrate.

fuging (2,500 G) for approximately two hours at 4° C., after which the layers became clear on standing at room temperature for a short time. Taking the nitrogen in the sample subjected to countercurrent fractionation as 100 per cent, the nitrogen distribution in the aqueous layers from tube 0 to tube 8 was as follows: 25, 16, 8.9, 5.9, 3.2, 1.9, 1.1, 0.95, and 0.75 per cent—total in aqueous layers, 63.7 per cent. The entire content of each tube was then dialyzed to remove the phenol. The resulting "endo" solutions contained a total of 26 per cent of the nitrogen in the original sample. The distribution of nitrogen from tube 0 to tube 8 was: 22.5, 11.1, 8.4, 6.5, 5.1, 4.3, 5.3, 9.9, and 26.9 per cent. Therefore, the separation of two fractions appeared to be quite sharp. The "endo" solutions from tubes 0 to 4 (aqueous end) were combined and concentrated for testing. Likewise, the "endo" solutions from tubes 5 to 8 (phenol end) were combined and concentrated for testing.

Bovine skin tests gave the following results:

	HOMOLOGOUS POTENCY	SPECIFICITY
Aqueous end	1.6	0.3
Phenol end	0.8	1.7

Countercurrent extraction, therefore, yielded a more potent but less specific material in the aqueous end and a less potent but more specific material in the phenol end.

A modified countercurrent extraction through four tubes was carried out in which the upper layer in each tube was concentrated Johne's bacilli culture filtrate rather than water. On separation and dialysis of the layers, only 14 per cent of the original nitrogen was recovered. Of the nitrogen recovered, the distribution in the "endo" solutions from the upper layers was as follows (from tube 0 to tube 3): 7.3, 9.4, 10.4, and 13.5 per cent. The corresponding figures for the "endo" solution from the lower layers were: 0.8, 2.1, 6.9, and 49.8 per cent. Therefore, the first extraction removed most of the nitrogen which could be extracted by phenol. The "endo" solution from the lower layer of the fourth tube was concentrated and tested. Its homologous potency was 0.8 and its specificity was 1.3.

TABLE 3

HOMOLOGOUS POTENCY AND SPECIFICITY OF CONCENTRATED JOHNIN BEFORE AND AFTER EXTRACTION WITH PHENOL

SAMPLE	HOMOLOGOUS POTENCY	SPECIFICITY
Concentration of johnin "endo" solution	0.63	0.70
First portion extracted with phenol	0.11	0.10
Second portion extracted with same phenol	0.13	0.11
Third portion extracted with same phenol	0.12	0.15
Fourth portion extracted with same phenol	0.12	0.18
Recovered from phenol extract	0.43	1.1

In a variation of countercurrent extraction in which a minimum amount of phenol was used, four 100 ml. portions of concentrated johnin were extracted successively with one 100 ml. portion of phenol. The aqueous layers were then washed with toluene to remove phenol while the phenol layer was frozen and stirred with 9 volumes of absolute alcohol containing a few drops of normal hydrochloric acid. The resulting precipitate was recovered and dissolved in water. The proportion of nitrogen remaining in each of the four aqueous residues was (from the first to the fourth): 50.3, 60.8, 75.2, and 76.3 per cent. The overall recovery of nitrogen was 80.5 per cent, of which five-sixths were in the aqueous solutions while one-sixth was recovered from the portion of phenol.

The results of these experiments are tabulated in table 3. As may be seen, a comparatively small amount of phenol results in the extraction of most of the more specific material from aqueous johnin.

Extraction of culture filtrate with phenol: Since the various countercurrent procedures effected separation of a more specific material from Johne's bacilli culture filtrate, a simple extraction was carried out, after which the phenol extract was fractionated by the addition of alcohol.

For this purpose 910 ml. of a concentrated "endo" solution of Johne's bacilli culture filtrate were extracted with three 200 ml. portions of phenol. The aqueous

residue, after being washed with ether, contained 79 per cent of the nitrogen in the original sample. The combined phenol extract was diluted with absolute alcohol while being stirred, but no precipitate appeared until the alcohol concentration was more than 30 per cent by weight. The first fraction of precipitate was taken at 50 per cent alcohol concentration by weight. This dark brown solid was washed first with alcohol, then with ether, dried, and dissolved; the yield was 3.5 per cent, based on the nitrogen in the original sample. The supernatant 50 per cent alcohol solution was made up to 80 per cent alcohol to obtain a second fraction, a brown, flocculent precipitate, which after being washed, dried, and dissolved accounted for 7.1 per cent of the nitrogen in the original sample.

The results of this fractionation are given in table 4. Here again the more specific material was extracted from johnin by phenol and, in this experiment, the extract was fractionated to yield at 80 per cent alcohol concentration a fraction which was considerably more specific than BAI regular johnin.

TABLE 4

HOMOLOGOUS POTENCY AND SPECIFICITY OF JOHNIN AFTER EXTRACTION WITH PHENOL AND OF THE FRACTIONS PREPARED FROM THE PHENOL EXTRACT

FRACTION	HOMOLOGOUS POTENCY	SPECIFICITY
Aqueous residue after extraction with phenol	0.11	0.09
Precipitated from phenol extract at 50 per cent alcohol concentration	0.48	0.34
Precipitated from supernatant at 80 per cent alcohol concentration	1.9	3.2

DISCUSSION

In previous papers the need for a more specific tuberculin was pointed out. The main point concerning tuberculin is that the BAI regular tuberculin currently in use, although being a uniform product prepared by a fairly simple procedure, cross-reacts with some heterologously sensitized animals. With regular johnin, prepared by essentially the same procedure as regular tuberculin except for the use of the proper organism, there is a lack of uniformity in addition to some of the undesirable cross-reactivity. That regular tuberculin is more uniform than regular johnin may be a result, at least in part, of the fact that the former is prepared on a larger scale, more flasks being pooled in the final product than in the production of the latter. Also the nonuniformity may be due to a difference in the amount of lysis which occurs in different batches of culture. A small pellicle of bacteria is planted in each flask; as multiplication occurs, some bacteria lyse, and in consequence the medium becomes enriched in the allergenic protein. Although the weight of dry bacteria is usually taken as the measure of growth, it is obvious that this may be quite unrelated to the amount of allergen in solution because the latter depends on the amount of lysis which takes place. Johne's bacilli cultures give greater yields of dry bacteria than *M. tuberculosis*, but the yield of protein material in the culture filtrate is far greater in the latter. There-

fore, simple concentration of the culture filtrate of *M. paratuberculosis* yields a rather nonuniform product because the product is prepared in small batches and the major part of the allergenic protein remains in the unlysed bacteria; a small change in the amount of lysis would make a great difference in the amount of protein in the relatively dilute protein solution. Hence, it is important in the preparation of johnin, even more than in the preparation of tuberculin, to purify, at least to some extent, the active material.

Of the procedures found useful in the preparation of a more specific tuberculin, several were found to be applicable to the preparation of a more specific johnin. However, fractionation by the addition of acid, which was so useful in the preparation of tuberculin, was not applicable in the fractionation of johnin. Therefore, the dilute culture filtrate was simply concentrated in a vacuum evaporator. The concentrate was then freed of small molecules by dialysis, and the "endo" solution was further concentrated prior to extraction with phenol. The use of fairly small volumes at this point facilitates the centrifugation and also decreases the amount of phenol required. On extraction of the aqueous solution with phenol, most of the nitrogen is left in the aqueous residue, but the more specific material is in the phenol extract. The active material can then be fractionated from the phenol extract by the addition of alcohol. The fraction precipitated at 50 per cent alcohol concentration is neither potent nor specific, but the second fraction, obtained at 80 per cent alcohol concentration, is more potent and more specific than BAI regular johnin. The yield of the more specific material is approximately 2.5 per cent, based on the nitrogen in the original culture filtrate. Although this yield appears to be very low, the greater part of the nitrogen lost was inactive or nonspecific material separated in the dialysis and extraction.

Since the best johnin fraction prepared was shown to be more specific than BAI regular johnin by tests on the skin of cattle artificially sensitized to *M. tuberculosis* and cattle sensitized to *M. paratuberculosis*, the fraction must now be tested on naturally sensitized animals in order to verify the enhanced specificity and also to determine whether the material is sufficiently specific for use in diagnostic work.

SUMMARY

The procedures utilized in the preparation of a more specific tuberculin have been applied to the preparation of a more specific johnin. Fractionation by the addition of acid is not applicable to johnin. A more specific johnin was prepared by the following steps: (*1*) concentration of the dilute culture filtrate; (*2*) dialysis; (*3*) extraction of the "endo" solution with phenol; and (*4*) fractionation of the phenol extract by the addition of alcohol, reserving the precipitate obtained at 50 to 80 per cent alcohol.

The yield of the final fraction is approximately 2.5 per cent based on the nitrogen in the original culture filtrate. According to comparative intradermal tests on cows artificially sensitized to *M. tuberculosis* and cows sensitized to *M. paratuberculosis*, the final fraction is more potent and more specific than Bureau of Animal Industry regular johnin.

SUMARIO

Estudios de la Fraccionación de la Tuberculina y la Johnina: III. Fraccionación de las Proteínas de la Johnina

Los procedimientos utilizados para la preparación de una tuberculina más específica han sido aplicados a la preparación de una johnina más específica. La fraccionación por la adición de ácido no es aplicable a la johnina. Se preparó una johnina más específica con los siguientes tiempos: (*1*) concentración del filtrado del cultivo diluído; (*2*) diálisis; (*3*) extracción de la solución de endo con fenol; (*4*) fraccionación del extracto fenólico por la adición de alcohol, reservando el precipitado obtenido con 50 a 80 por ciento de alcohol. El rendimiento de la fracción final es aproximadamente de 2.5 por ciento, a base del nitrógeno contenido en el primitivo filtrado del cultivo. De acuerdo con intradermorreacciones comparadas en vacas sensibilizadas artificialmenta al *M. tuberculosis* y en vacas sensibilizadas al *M. paratuberculosis*, la fracción final es más potente y más específica que la johnina regular de la Oficina de Industria Animal de los E. U. A.

RESUME

Etudes sur le fractionnement de la tuberculine et de la johnine: III. Fractionnement des protéines de la johnine

Ce procédé utilisé dans la préparation d'une tuberculine d'un degré de spécificité plus élevé a été appliqué à la préparation d'une johnine plus spécifique. Le fractionnement par addition d'acide n'est pas applicable à la johnine. Les temps de la préparation d'une johnine plus spécifique sont les suivants: 1) concentration du filtrat de la culture diluée; 2) dialyse; 3) extraction de l'endo-solution par le phénol; 4) fractionnement de l'extrait phénolique par addition d'alcool, en réservant le précipité obtenu á 50 á 80% d'alcool. Le rendement de la fraction finale est approximativement de 2,5%, en prenant pour base l'azote du filtrat de la culture originale. Conformément aux tests intradermiques comparatifs effectués sur des vaches sensibilisées artificiellement au *M. tuberculosis* et chez des vaches sensibilisées au *M. paratuberculosis*, la réaction finale est plus forte et plus spécifique que celle donnée par la Johnine régulière du *Bureau of Animal Industry*.

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EFFECT OF BCG VACCINATION ON MICE INFECTED WITH TUBERCULOSIS[1]

FLORENCE K. FITZPATRICK

(Received for publication March 23, 1953)

INTRODUCTION

As a rule, BCG vaccination has been applied to patients who were found to be negative to a preliminary tuberculin skin test. Some investigators, however, have considered preliminary skin testing to be unnecessary and BCG vaccination to be harmless in positive reactors (1, 2, 3). Ustvedt (4) recently pointed out that the question of possible harm in vaccinating persons with a positive cutaneous reaction to tuberculin is still an open one. Calmette himself stated that, in reactors to tuberculin, inoculation with BCG caused the development of a Koch phenomenon at the site of injection. When large-scale vaccination programs must be undertaken, it would mean a great saving of time and personnel if vaccination could be carried out safely without preliminary tuberculin testing. Experimental work along these lines has been carried out in man and in guinea pigs by French workers (5, 6, 7). Some of these investigators came to the conclusion that inoculation of infected subjects with BCG was harmless, while others noted an aggravating influence with large doses. Differences in routes of administration and size of doses probably account for some of the disagreement. In an attempt to reconcile opposing views on the question, the effect of vaccination with BCG on mice already infected with tuberculosis has been studied in this laboratory.

METHODS AND MATERIALS

Female mice of the CF_1 strain (Carworth Farms) were used. They were housed in an air-conditioned room at a temperature of 70 to 74°C. with 60 per cent relative humidity. Vaccine was administered by the subcutaneous route to groups of 10 mice, the vaccine consisting of the BCG (Phipps) strain of tubercle bacillus grown in Dubos medium (8) for five to seven days at 37°C. The mice were infected intravenously with 0.25 ml. of a five-day culture of the H37Rv strain, also grown in Dubos medium. The quantity of vaccine or infectious material was estimated routinely by readings on the Klett-Summerson colorimeter. (It was found that seven-day cultures giving a Klett reading of 100 contain 1.2 mg. of organisms per ml., dry weight.)

As a control on the efficacy of the vaccine, several lots of mice were vaccinated one to three weeks prior to infection with a single dose. For the study of the effect of vaccination on tuberculous disease, mice were vaccinated on the day of infection, or one week after, or two weeks after, infection. All animals were fed Rockland diet ad libitum and observed until at least 50 per cent died, at which time some were examined in order to establish tuberculosis as the cause of death.

RESULTS

Vaccine when given before infection gave a striking increase in survival time, as has been reported by others working with mice (9, 10). The dose required to

[1] From the Department of Virology, Research Division, Sharp & Dohme, Incorporated, West Point, Pennsylvania.

obtain this result is relatively much larger than that customarily given to human beings. In the present experiments, in which a single vaccine dose was used, all of the mice died eventually, the length of survival being dependent upon the quantity of vaccine administered and the severity of subsequent infection. When BCG was given after infection, small amounts of vaccine (0.05 to 0.2 mg. per mouse) insufficient to effect a notable increased survival in normal mice, given on the day of infection or at later stages, did not aggravate the disease. Large doses of vaccine, on the other hand, (0.3 to 1.2 mg.) accelerated the disease whether given on the day of infection or at a later time. The quantities of vaccine which produced this effect were greatly in excess of the usual 0.1 mg. dose used for human immunization. In figures 1 and 2 the percentages of survivals of

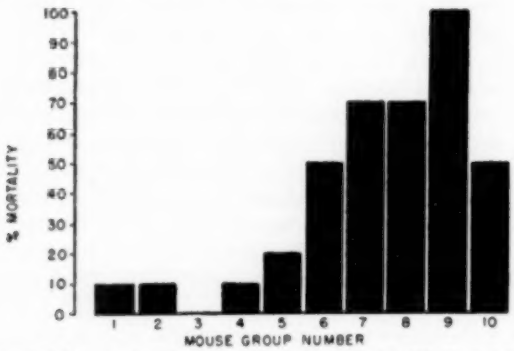

FIG. 1. Effect of BCG vaccination on mice infected with H37Rv* (chronic infection—large doses of vaccine).

Groups 1 through 5 vaccinated before infection with 0.5 to 1.0 mg.
Group 6 vaccinated on day of infection with 1.0 mg.
Groups 7 and 8 vaccinated one week after infection with 1.0 and 0.5 mg.
Group 9 vaccinated two weeks after infection with 1.0 mg.
Group 10 unvaccinated infected controls.

* Per cent mortality when controls showed 50 per cent deaths.

vaccinated mice at the time when 50 per cent of unvaccinated controls had died are shown.

COMMENT

Although it goes without saying that results in the relatively resistant mouse do not necessarily parallel what might occur in humans, it would appear on the basis of the mouse experiments cited that the manifestation of clinical disease in patients soon after BCG immunization should not cause greater concern than if they had not been vaccinated. In some cases reported in the European literature in which vaccination was considered an aggravating influence, the patients were given BCG by the scarification method. In the present mouse experiments, the subcutaneous route was used and three to twelve times the weight of micro-

organisms employed in human immunization were required to shorten the survival of mice previously infected.

An attempt has been made by experimental means to harmonize different points of view on the question of whether it is or is not harmful to vaccinate with BCG in the presence of tuberculous infection. If the results obtained in mice can be applied to man, it would appear that vaccination with BCG as usually practiced should do no harm when performed on human beings during the incubation period of tuberculous disease.

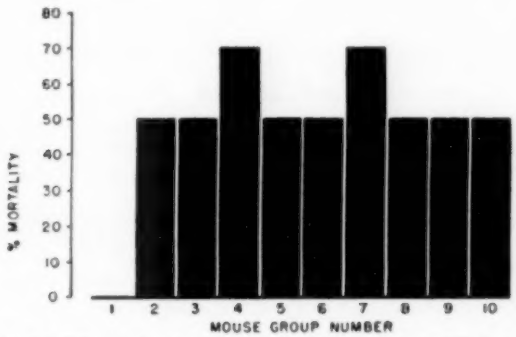

FIG. 2. Effect of BCG vaccination in mice infected with H37Rv* (acute infection—large and small doses of vaccine).

Group 1 vaccinated before infection with 0.9 mg.
Groups 2 and 3 vaccinated on day of infection with 0.2 and 0.1 mg.
Groups 4, 5, and 6 vaccinated one week after infection with 0.35, 0.17, and 0.08 mg.
Groups 7, 8, and 9 vaccinated two weeks after infection with 0.3, 0.15, and 0.07 mg.
Group 10 unvaccinated infected controls.

* Per cent mortality when controls showed 50 per cent deaths.

SUMMARY

Mice were inoculated with varying doses of BCG at zero, one, two, or three weeks after infection with virulent human tubercle bacilli and their survival times were compared with those of unvaccinated controls. It was found that small quantities of vaccine did not influence the outcome of the infection but that doses in excess of amounts needed to produce significant survival in normal mice accelerated the disease.

SUMARIO

Efecto de la Vacunación BCG en los Ratones Infectados con Tuberculosis

Después de infectarlos con bacilos tuberculosos humanos virulentos, se inoculá a ratones con dosis variables de BCG a plazos de cero y una, dos y tres semanas, comparándose los respectivos tiempos de sobrevivencia con los de testigos sin vacunar. Observóse que pequeñas cantidades de vacuna no afectaban la evolución de la infección, pero que dosis superiores a las necesarias para producir una sobrevivencia de importancia en los animales normales aceleraban la dolencia.

RESUME

Résultat de la vaccination par le BCG dans l'infection tuberculeuse expérimentale de la souris

L'inoculation de doses variées de BCG a été pratiquée, chez des souris, immédiatement et une, deux ou trois semaines après l'infection expérimentale effectuée avec des bacilles tuberculeux humains virulents. Le temps de survie a été comparé avec celui enregistré pour les animaux témoins, non vaccinés. Il a été observé qu'en petite quantité le vaccin ne modifiait pas l'issue de l'infection, mais que les doses supérieures aux quantités nécessaires pour obtenir un temps de survie significatif, chez la souris normale, accélérait l'évolution de l'affection.

Acknowledgment

We are indebted to Miss Stella Pietryk for valuable technical assistance.

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Case Reports

APLASTIC ANEMIA FOLLOWING THE USE OF STREPTOMYCIN AND PARA-AMINOSALICYLIC ACID[1]

Report of a Fatality

HENRY W. WALTERS

(Received for publication April 16, 1953)

The administration of streptomycin and para-aminosalicylic acid (PAS) together has been used in the treatment of all forms of tuberculosis since 1948. Although treatment has been prolonged in many patients, serious complications have been relatively few. In the cases which have shown evidence of bone marrow depression, immediate cessation of chemotherapy and early treatment of the disturbed hemopoiesis has resulted in early recovery. The present report is concerned with a patient who developed a fatal aplastic anemia while receiving streptomycin-PAS therapy.

CASE REPORT

A 69-year-old white male had been hospitalized continuously since November 22, 1924, for the treatment of a schizophrenic reaction of the catatonic type. He had a simple pertrochanteric fracture of the left hip in December of 1944. He developed severe jaundice beginning July 16, 1941. The icterus index was 75; the van den Bergh delayed test was 4.0 units and the immediate was 6.0 units. The erythrocyte count was 3.9 million per cu. mm.; the leukocyte count was 6,200, with a differential distribution of 67 per cent polymorphonuclear leukocytes, 32 per cent lymphocytes, and 1 per cent monocytes; the hemoglobin was 78 per cent; the coagulation time was three and one-half minutes. Because of a diagnosis of obstructive jaundice, probably due to carcinoma of the head pancreas, a laparotomy was performed on September 26, 1941. No abnormalities were detected at operation. The jaundice began clearing soon after the operation and had entirely disappeared by March of the following year.

Moderately advanced pulmonary tuberculosis was discovered on February 20, 1947. Because of the persistence of the tuberculous lesions, chemotherapy (streptomycin, 1 gm. twice weekly, and PAS, 4 gm. three times daily) was initiated on July 6, 1952, and no other medication was prescribed.

The patient showed no intolerance to the drugs until October 15, 1952, when he refused to eat or take PAS, stating he felt pregnant. During the night his temperature reached 104.4°F. (rectal); pulse, 140. A leukocyte count (October 16, 1952) revealed 4,000 cells per cu. mm., predominantly mononuclear cells. The previous blood count had been taken on September 29, 1952, and was erythrocytes, 3.9 million per cu. mm.; hemoglobin, 13.2 gm.; leukocytes 7,600 (74 per cent neutrophils, 20 per cent lymphocytes, 2 per cent monocytes, and 4 per cent eosinophils). Streptomycin and PAS were discontinued, and forced feeding was started. Penicillin in large doses, B-6, and pentose nucleotides were administered daily.

The hematologic findings until the time of death were as follows:

Date	*Erythrocyte*	*Hemoglobin*	*Leukocyte*
September 29	3.9 million	13.2 gm.	7,600
October 16			4,000 (predominantly mononuclear cells)
October 17	3.75 million	13.0 gm.	600
October 20	3.70 million	11.0 gm.	400 (mostly mature lymphocytes)
October 22	2.47 million	5.0 gm.	150

[1] From the Tuberculosis Service, Veterans Administration Hospital, St. Cloud, Minnesota.

The bromsulfalein test on October 17 showed 6 per cent retention in thirty minutes.

The phenolsulfonphthalein test on October 17 showed 50 per cent excretion at one hour and 80 per cent excretion at two hours.

The urine was normal until October 17, 1952, when examination revealed 3-plus albumin with a specific gravity of 1.030, an occasional leukocyte, erythrocyte, and cast. On October 20, 1952, the urine was acid, with a specific gravity of 1.010 and a 4-plus albumin.

The patient's temperature ranged from 102° to 105°F. (rectal) until the day of death (October 22, 1952), when it rose to 107°F. (rectal). The pulse range was 120 to 160; and the respirations, 30 to 50 per minute. Unfortunately, blood transfusions were not employed until approximately twelve hours before death.

At autopsy, the pathologic diagnoses established were pneumonia, lobular, left lower lobe; pulmonary edema and hemorrhage, bilateral; tuberculosis, pulmonary, right apex; pleuritis, fibrous, right; cerebrovascular congestion, petechial hemorrhages, minimal subarachnoid hemorrhage; generalized dermal petechiae; and passive congestion of liver, spleen, and kidneys.

Post-mortem studies of bone marrow revealed no erythrocytes, no megaloblasts, and very few leukocytes (mostly lymphocytes and plasma cells). On the basis of these findings, a diagnosis of aplastic anemia was made.

The pulmonary tuberculous lesions consisted of a nodular necrotic mass 3 cm. in diameter and a cavity 2 cm. in diameter, both located in the apical segment of the right upper lobe.

COMMENT

Although serious complications are infrequent in the treatment of tuberculosis with the chemotherapeutic agents currently employed, this case is a reminder that they may occur. Since there is a trend to treat tuberculous patients with chemotherapy on an outpatient basis, one must be alert to the possibility of bone marrow depression. Psychotic patients may not be able to call attention to the symptoms and early signs of the condition and, therefore, need to be carefully observed.

SUMMARY

A case of aplastic anemia which terminated in death occurred in a tuberculous psychotic patient approximately three months after the beginning of long-term chemotherapy consisting of intermittent streptomycin sulfate and daily para-aminosalicylic acid. Which one of the two drugs was responsible could not be established. The case is reported as a reminder that serious complications can occur in the treatment of tuberculosis with long-term chemotherapy.

SUMARIO

Anemia Aplásica Consecutiva al Uso de Estreptomicina y Acido Para-Aminosalicílico: Comunicación de una Muerte

Un caso de anemia aplásica que culminó en la muerte fué observado en un psicópata tuberculoso uno tres meses después de comenzar una quimioterapia a largo plazo, compuesta de sulfato de estreptomicina intermitentemente y de ácido para-aminosalicílico a diario. No pudo establecerse cuál de las drogas fué la causante. Preséntase el caso para hacer recordar que pueden ocurrir complicaciones graves en el curso de la quimioterapia a largo plazo de la tuberculosis.

RESUME

Anémie aplastique à la suite de l'emploi de la streptomycine et de l'acide para-aminosalicylique: Rapport sur un décès

Un cas d'anémie aplastique, qui a eu une issue fatale, s'est produit chez un tuberculeux affecté de psychose environ un mois après l'instauration d'une chimiothérapie à long terme,

comportant une cure intermittente de streptomycine avec administration quotidienne d'acide para-amino-salicylique.

Il n'a pas été possible de déterminer quelle était celle des deux substances responsable de cette réaction. Ce cas est rapporté pour rappeler que, dans le traitement de la tuberculose, des complications graves peuvent se produire, au cours de la chimiothérapie prolongée.

Acknowledgments

The author acknowledges with gratitude the assistance of Dr. P. E. Stangl, St. Cloud, Minnesota, and Dr. Arthur C. Aufderheide, Minneapolis, Minnesota, for the pathologic diagnoses and the bone marrow tissue studies.

PERIPHERAL NEUROPATHY IN TUBERCULOSIS PATIENTS TREATED WITH ISONIAZID[1]

HAROLD N. LUBING

(Received for publication May 6, 1953)

INTRODUCTION

Severe toxic reactions to therapeutic doses of the antituberculous drug isoniazid are regarded as uncommon. The untoward reactions first reported by Robitzek and Selikoff (1) have proved to be infrequent and minor in importance. During the course of isoniazid treatment of more than 170 patients in the Veterans Administration Hospital, Madison, Wisconsin, in the past twelve months, it has not been necessary to discontinue therapy because of rash, liver damage, gastrointestinal intolerance, or urinary retention. Restlessness, irritability, and hyperreflexia occasionally have been noted. No evidence of serious central nervous system hyperirritability has been encountered. Nevertheless, 6 patients in this series have developed manifestations of peripheral neuropathy. This condition is noteworthy because the symptoms may become severely distressing and require the discontinuation of isoniazid.

CASE REPORTS

Case 1. T. E. is a 47-year-old single white male who entered the hospital on March 11, 1952, with the diagnosis of far advanced active pulmonary tuberculosis of fourteen months' duration. He received extensive treatment with streptomycin and para-aminosalicylic acid (PAS) without improvement and, therefore, was placed on isoniazid, 50 mg. three times daily, on March 20, 1952. After one month, the dose was increased to 150 mg. three times daily (approximately 9.2 mg. per kg.). Two weeks later, he developed a papular rash over the forehead, and a few days later began complaining bitterly of "slivers" and numbness in his hands and legs. These symptoms became more marked even though the dosage was reduced to 50 mg. three times daily on May 22. He was given nicotinic acid, 50 mg. three times daily, without benefit. Vitamin B complex was then substituted and continued for two months without alleviation of symptoms. On August 6, the patient was started on parenteral vitamin B_{12}, 60 mg. three times weekly. When no improvement resulted, it was necessary to discontinue the isoniazid. The patient, thereafter, reported that he felt somewhat improved although some numbness and tingling continued. Progression of the patient's pulmonary tuberculosis prompted reinstituting isoniazid, 50 mg. three times daily, on September 24. This has been continued in spite of discomfort from his peripheral neuropathy. Neurologic examination revealed that this patient had motor weakness of the hands and feet, glove-stocking type of hypesthesia to light touch with hyperesthesia to pin prick. Vibratory and position senses were normal. Deep reflexes were diminished in the upper extremities and lost in the lower extremities.

Case 2. K. E. is a single white male who entered the hospital as a "transfer patient" on December 21, 1951, with the diagnosis of far advanced active pulmonary tuberculosis of eight and one-half years' duration. He had exhibited psychotic behavior in the past, which was classified as a schizophrenic reaction, paranoid type. He had received several courses of streptomycin and para-aminosalicylic acid, and his tubercle bacilli were highly resistant to both of these agents. The patient was placed on isoniazid, 50 mg. three times daily, on

[1] From the Psychosomatic Section of the Medical and Tuberculosis Service, Veterans Administration Hospital, Madison, Wisconsin.

March 20, 1952. The dosage was increased to 150 mg. three times daily on April 20, 1952 (approximately 7.2 mg. per kg.). He first complained of "slivers" in his hands and feet on June 2, 1952. Increasing severity of his complaints led to a trial on vitamin B_{12}, 1,000 γ four times weekly. This was discontinued after two weeks because no improvement was noted. Although the symptoms continued unabated, the patient was maintained on isoniazid because of the critical condition of his pulmonary tuberculosis. He was transferred to another hospital on January 19, 1953, still having symptoms and signs of peripheral neuropathy.

Case 3. M. J. was a 57-year-old single white male who entered the hospital on November 13, 1951, with the diagnosis of far advanced active pulmonary tuberculosis of five years' duration. This patient was seriously ill at the time of admission, and his prognosis was considered to be very grave. During the initial period of hospitalization, he received streptomycin and para-aminosalicylic acid, as well as Terramycin, without benefit. On March 20, 1952, he was placed on isoniazid, 50 mg. three times daily. This was increased to 150 mg. three times daily (approximately 8.6 mg. per kg.) on April 25, 1952. Abdominal discomfort and nausea developed in May. On approximately July 15, he began complaining of "soreness like a toothache in both feet around the toes and balls of the feet." There were no objective neurologic changes. Isoniazid was discontinued on July 25, and he was given vitamin B_{12}, 15 γ intramuscularly, daily for two weeks without benefit. Thiamine, 100 mg. three times daily after meals, then was given for two weeks along with nicotinic acid, 25 mg. three times daily for a few days. Although the patient felt somewhat improved, he continued to complain of pain in the feet. His general condition deteriorated. The isoniazid, 50 mg. three times daily, was reinstituted on September 5, 1952. The peripheral neuropathy continued unchanged until the patient's death on November 28, 1952. The post-mortem diagnoses were: far advanced pulmonary tuberculosis, bilateral; empyema (right); focal tuberculosis of the spleen; cholesithiasis; portal cirrhosis.

Case 4. L. G. is a 46-year-old married white male who was admitted to the hospital on June 30, 1952, with the diagnosis of far advanced active pulmonary tuberculosis of two years' duration. Isoniazid, 100 mg. three times daily (approximately 6.8 mg. per kg.), was prescribed on July 14, 1952. Seven weeks later, he developed pain and burning of the soles of his feet, "as if wearing a new pair of shoes." He was placed on vitamin B complex with only moderate relief of symptoms. He left the hospital against medical advice on December 22, 1952, at which time he still had mild discomfort in his feet. Neurologic findings were limited to slight hypesthesia in the extremities and diminution of knee and ankle jerks.

Case 5. S. E. is a 58-year-old widowed white male who entered the hospital on November 1, 1951, with far advanced active pulmonary tuberculosis complicated by genitourinary tuberculosis of six months' duration. He was placed on streptomycin and para-aminosalicylic acid. On March 14, 1952, approximately two weeks following bilateral epididymectomy, he developed tuberculous meningitis. Isoniazid, 50 mg. three times daily, was started on March 19. This was increased to 150 mg. three times daily (approximately 8.2 mg. per kg.) on April 2, 1952.

The patient gradually improved but then sustained a left hemiplegia on June 19, 1952. In February, 1953, he began complaining of pain in the hands and feet "as though the skin is cracking." Vitamin B_{12}, 30 γ daily, was given without much improvement. Neurologic examination showed residual hemiplegia on the left, and right facial weakness, atrophy of the hand muscles bilaterally with weakness of grip on the right. General reduction of sensation was present on the left side. Hypersensitivity to pin prick was noted over the right hand and foot. No abnormal reflexes were noted. The neurologic picture was complicated by the previous meningitis and hemiplegia. Peripheral neuropathy was considered to be present because of the persistent complaint of "soreness" of the hands and feet.

Case 6. L. C. is a 41-year-old divorced white male who was admitted to the hospital September 11, 1952, with the diagnosis of moderately advanced active pulmonary tuberculosis of two years' duration. An additional diagnosis of panhypopituitarism was established on admission to the hospital. He was started on streptomycin, 1 gm. twice weekly, and para-aminosalicylic acid, 12 gm. daily. He was intolerant to para-aminosalicylic acid. This was discontinued and replaced by isoniazid, 100 gm. three times daily (approximately 3.9 mg. per kg.), on September 29, 1952. During the first week of November, 1952, the patient began to complain of pain and numbness in his hands and feet, gradually spreading upward toward the elbows and knees. Decreased response to painful stimuli was noted, but there was no change in light touch sensation. The deep reflexes were all markedly diminished. The isoniazid was reduced to 50 mg. three times daily on November 27, 1952, and vitamin B_{12} was first administered in a dosage of 1,000 γ daily for six days and then 30 γ daily for twenty-one days. It finally became necessary to stop the isoniazid on December 17. The patient improved, and isoniazid, 50 mg. three times daily, was again started. This was discontinued after eleven days, when his discomfort again became intolerable. On February 14, 1952, vitamin B_{12}, 1,000 γ, and thiamine, 50 mg. three times daily, were given and continued for seven days. The vitamin B_{12} was then reduced to 30 γ daily. Subsequently, his symptoms have abated somewhat, but the neurologic findings have remained unchanged.

DISCUSSION

Although peripheral neuropathy was not found in a large number of tuberculous patients who received isoniazid on the dosage of 50 to 100 mg. three times daily, it occurred in 6 patients who received this drug in the higher dosage of 100 to 150 mg. three times daily (3.9 to 9.2 mg. per kg.). Five of these 6 patients had far advanced pulmonary tuberculosis. All of this group were chronically ill, toxic, and emaciated. Within six weeks to eleven months after initiating isoniazid therapy, these patients began complaining of pain and parasthesias in the extremities. Neurologic findings included weakness in the muscles of the hands, impaired dorsiflexion of the feet, diminished sensory modalities, such as light-touch, vibratory and position sense, and reduction or absence of deep reflexes, particularly the knee and ankle jerks. Isoniazid was discontinued, at least temporarily, in most of these cases and the symptoms and signs slowly abated. Reinstitution of the drug generally was followed by exacerbation of symptoms.

Vitamin B_{12} in moderate and even massive doses did not appear to modify the rate of improvement. Likewise, nicotinic acid, vitamin B, and vitamin B complex had no significant effect on the intensity of the symptoms.

SUMMARY

The cases of 6 patients with pulmonary tuberculosis who developed symptoms and signs of peripheral neuropathy while receiving isoniazid therapy are reported.

This syndrome occurred only in patients who were receiving higher dosages of isoniazid.

The symptoms were sufficiently severe to necessitate temporary discontinuation of drug therapy in most cases.

There was only slow symptomatic improvement with interruption of drug treatment. Intensive vitamin therapy, including vitamin B_{12}, proved to be ineffective.

SUMARIO

Neuropatía Periférica en Tuberculosos Tratados con Isoniacida

Los casos presentados comprenden 6 enfermos con tuberculosis pulmonar que manifestaron síntomas y signos de neuropatía periférica mientras recibían la isoniacidoterapia.

Ese síndrome no apareció más que en los enfermos que recibían las dosis más altas de isoniacida.

Los síntomas alcanzaron suficiente gravedad para obligar a abandonar temporalmente la farmacoterapia en la mayoría de los casos.

Al interrumpirse la medicación, sólo se observó mejoría sintomática lenta. La vitaminoterapia intensa, comprendiendo vitamina B_{12}, resultó ineficaz.

RESUME

Neuropathie périphérique chez des tuberculeux traités par l'isoniazide

Publication des observations de six cas de tuberculose pulmonaire présentant des signes de neuropathie périphérique au cours d'un traitement par l'isoniazide.

Ce syndrome ne s'est produit que chez les malades recevant les doses d'isoniazide les plus élevées.

Dans la plupart des cas, les symptômes étaient suffisamment sévères pour nécessiter l'interruption temporaire de l'isoniazide.

L'amélioration générale a été ralentie quand le traitement a été discontinué.

Une vitaminothérapie comportant la vitamine B_{12} s'est montrée inefficace.

REFERENCE

(1) Robitzek, E. H., and Selikoff, I. J.: Hydrazine derivatives of isonicotinic acid hydrazide (Rimifon, Marsilid) in the treatment of active progressive caseous-pneumonic tuberculosis: A preliminary report, Am. Rev. Tuberc., 1952, *65*, 402.

Notes

PRESENT STATUS OF BCG STUDIES[1]

At the annual meeting of the National Tuberculosis Association three years ago, Dr. Robert Anderson[2] discussed the attitude of the Public Health Service toward BCG vaccination. He pointed out the wide differences of opinion on many questions about BCG and its use; the recurrent theme throughout his talk was how little was really known about BCG—how little solid evidence there was amid all the claims and counterclaims of its value.

Because of this, Dr. Anderson said, the Public Health Service could recommend the use of BCG only for selected groups with known high risk of exposure to tuberculous infection. Tuberculosis had been declining sharply for many years and there was no reason to think that it would not continue to decline, perhaps even more rapidly. Moreover, it was not clear that the advantages of using BCG would outweigh the disadvantages of losing the tuberculin test as a diagnostic and case-finding tool. It was believed that mass vaccination programs in this country could be justified only for the purpose of making carefully documented studies to determine the value of BCG in tuberculosis control.[3]

That was not a recommendation to be made lightly, in the face of strong popular belief in BCG and its widespread use abroad. It was not a recommendation which could be made without an obligation to make known any indication that it should be changed. Nevertheless, about the only reason for changing the recommendation would be decisive evidence of the effectiveness of BCG in human beings. A number of groups in different parts of the world, including the Public Health Service, are actively trying to find such evidence. It is the purpose of this report to describe very briefly the several large-scale evaluation studies undertaken by the Service and to present the first preliminary results of these studies.

In planning the studies, decisions were required on several critical issues.

> Of primary importance was the conviction that it was absolutely necessary to have control studies in which vaccination would be deliberately withheld from a randomly selected part of the population found eligible for vaccination. The effectiveness of BCG would be measured by comparing the rate of tuberculous infection in the two comparable groups, the one vaccinated, the other not vaccinated. An alternative, to vaccinate all who were eligible and expect to measure the effect of vaccination by the change in tuberculosis morbidity and mortality, was rejected as unsound. Similarly, it was unacceptable to vaccinate all who were willing to be vaccinated and use those who refused as controls.
>
> Another important decision concerned the size of the studies. With the steady drop in the prevalence of tuberculosis, only in large studies would there be enough cases of tuberculosis to indicate whether there was less tuberculosis among the vaccinated than among the controls.
>
> This aspect led to a third, the matter of evaluation. Few people today have the time, the staff, or the money to do regular, periodic examinations of large groups of

[1] Presented before the Medical Session, as part of the session on *Tuberculosis Control*, at the annual meeting of the National Tuberculosis Association, Los Angeles, California, on May 20, 1953.

[2] Anderson, R. J., and Palmer, C. E.: J.A.M.A., 1950, *143*, 1048.

[3] Anderson, R. J.: Pub. Health Rep., August 4, 1950.

apparently healthy individuals. Evaluation therefore would have to depend primarily on the established systems of finding and reporting tuberculosis cases and deaths.

Finally, in order to determine the impact of the BCG program on the total tuberculosis problem, it would be necessary to know about the tuberculosis which occurred, not only among the vaccinated and controls, but also among the tuberculin reactors who were ineligible to receive BCG.

Thus, it was determined that the main characteristics of the studies should be: first, that they would be control studies (some people would be vaccinated, some not), second, that they would be very large-scale studies; third, that follow-up would be made an integral part of the established system of notification of tuberculosis cases and deaths; fourth, that both those eligible for vaccination and those not eligible would be included in the follow-up.

Such studies, it was fully recognized, might be criticized because the evaluation depends so heavily on mortality and morbidity reporting. However, just such information is used to measure all our efforts in tuberculosis control. After all, if BCG vaccination in a population is not effective enough to be recognized through reasonably efficient reporting of tuberculosis cases and deaths, its widespread use can hardly be advocated.

The first study was started in 1947 in Muscogee County, Georgia, a community with a population of approximately 100,000 persons. In the spring of 1947, the entire school population was tuberculin tested, and half of the nonreactors were vaccinated. Three years later, in 1950, the BCG program was made part of a mass survey of the whole population, who were invited to have chest roentgenograms, tuberculin tests, and BCG vaccination. Approximately 10,000 schoolchildren were included in the early program; about 60,000 of the general population were added in 1950. Muscogee County is a reasonably typical community with a tuberculosis mortality rate somewhat below that of the rest of the country. The program there is directed by a full-time Public Health Service physician who, in cooperation with the practicing physicians, is able to ensure accurate diagnosis and efficient reporting of cases and deaths from tuberculosis.

The second Public Health Service program is among the American Indians, where the prevalence of tuberculosis is many times higher than in the rest of the country. The field work of this cooperative undertaking was carried out in 1949 by the Bureau of Indian Affairs and included a high proportion of the Indian children attending federal and mission schools in the continental United States. The number of children under study is 27,000.

The third program is in Puerto Rico, where the field work of testing and vaccinating was begun in the fall of 1949 and completed in the spring of 1951. It is a cooperative undertaking with the Insular government and was offered to all of the estimated 400,000 children attending school on the island. Although the field teams visited almost every school on the island, only about one-third of the expected number of children actually participated. Even so, it was expected that the high tuberculosis mortality rate in Puerto Rico might give, fairly soon, an indication of what BCG could do. The total number of children under study is 165,000, including 18,000 pre-school children.

A fourth study was undertaken in a special group, 20,000 mental patients of all ages in state institutions in Ohio.

In table 1 is shown the subdivision of each study population into three groups: the tuberculin reactors, the vaccinated, and the controls.

In the first three studies, about half of the populations were tuberculin reactors and not eligible for vaccination, leaving the other half to be divided into the vaccinated and

control groups. The division, however, was different in each study: in Georgia, one-half of the persons were vaccinated; in Puerto Rico, 2 of 3, and among the Indians, 3 of 4. Of the 20,000 Ohio mental patients, three-fourths were already reactors, leaving just over 5,000 eligible for vaccination. In this group, 6 of 7 were vaccinated.

The total number of persons included in the four studies is 282,000: 87,500 vaccinated, 50,500 controls, and 144,000 tuberculin reactors. The nonreactors were subdivided into vaccinated and control groups by strictly unbiased methods which ensure that the two groups are entirely comparable. The stage has thus been set for several large-scale tests of what BCG could accomplish as a broad public health measure.

TABLE 1

LOCATION AND SIZE OF THE BCG CONTROL STUDIES OF THE PUBLIC HEALTH SERVICE

YEAR PROGRAM STARTED	LOCATION AND TYPE OF STUDY GROUP	PERSONS	VACCINATED GROUP	CONTROL GROUP	TUBERCULIN REACTORS
	Muscogee County, Georgia				
1947	Schoolchildren	10,000	2,500	2,300	5,200
1950	General population	60,000	16,000	16,000	28,000
1949	American Indian schoolchildren	27,000	11,500	3,500	12,000
1950	Puerto Rican children	165,000	53,000	28,000	84,000
1948	Ohio mental patients	20,000	4,500	700	14,800
Total		282,000	87,500	50,500	144,000

TABLE 2

TUBERCULOSIS CASES AND DEATHS REPORTED BY DECEMBER, 1952, AMONG PERSONS IN PUBLIC HEALTH SERVICE BCG CONTROL STUDIES

STUDY GROUP	RELATIVE SIZE OF SUBGROUPS V:C:R	TYPE OF REPORT	VACCINATED GROUP	CONTROL GROUP	REACTORS
Muscogee County, Georgia, population	1:1:2	Deaths	0	0	3
		Cases	2	3	20
American Indian schoolchildren	3:1:3	Deaths	0	1	13
Puerto Rican children	2:1:3	Deaths	1	4	24
		Cases*	(6)	(2)	(75)
Ohio mental patients	6:1:20	Deaths	4	1	36
		Cases	43	6	286

* Utilizing only a limited set of reports.

It has been no small task to carry the studies this far—to tuberculin test and vaccinate, to make accurate individual records, and to set up the necessary statistical files or rosters. Now, these studies are in the follow-up phase, matching current tuberculosis mortality and morbidity reports with the rosters and, in so far as possible, checking the accuracy of these reports.

The results of the follow-up thus far are summarized in table 2. In some respects they are both striking and informative. All four studies show that a very large part of the tuberculosis appearing in the study groups has occurred among those who were already

infected, i.e., the tuberculin reactors who were not eligible for vaccination. In other respects, the results are inconclusive. Little tuberculosis has appeared in those eligible for vaccination, whether or not they had been vaccinated.

In Muscogee County there have been only 3 deaths from tuberculosis so far in the entire study population, all of them among the tuberculin reactors. The morbidity has also been remarkably low. Only 25 new cases of tuberculosis have occurred: 20 in the three years of follow-up of the older group, and 5 in the six years of follow-up of the original school group. The bulk of the new cases has occurred among those not eligible for vaccination; very little tuberculosis has appeared among the nonreactors. The 5 cases among the vaccinees and controls have occurred in the large group who entered the study in 1950; there have been no cases of tuberculosis in six years among either the vaccinated or control schoolchildren.

Results from the Indian study are limited to information on deaths. There have been 13 tuberculosis deaths among the 12,000 tuberculin reactors. Only one tuberculosis death has been reported for the 3,500 controls. If this rate were applied to the vaccinated, 3 deaths would be expected, while actually there was none. No conclusions can be drawn from so few cases, but the results do not disagree with the view that vaccination is of value.

In Puerto Rico also there has been a considerable concentration of tuberculosis mortality in the tuberculin reactors, 24 deaths during the first few years of the program. In this study, however, there is a little stronger indication that BCG may be of value. There have been 4 deaths in the controls and only one in the vaccinated group, which contained twice as many children. If the mortality rate of the controls were applied to the vaccinated, 8 deaths would be expected, but only one occurred. The numbers, of course, are very small, but they do suggest that the vaccinated are faring better than the controls.

Tuberculosis morbidity reports in Puerto Rico are somewhat deficient in the completeness of the reporting and the accuracy of diagnosis, but there is no reason to suspect any bias related to the fact of BCG vaccination. For this progress report, only a very limited number of morbidity reports was available. They show 6 new cases of tuberculosis among the vaccinated, 2 among the controls, and 75 among tuberculin reactors. Here, again, most of the cases have occurred among the reactors. Unlike the mortality data, the morbidity material does not show signs that the vaccinated have been benefited by their vaccination.

Among Ohio mental patients there is essentially no difference between the vaccinated and controls with respect to tuberculosis mortality. One death occurred among the 700 controls, and 4 among the 4,500 who had been vaccinated. As in the other studies, the great majority of the deaths from tuberculosis have occurred among the tuberculin reactors.

New cases of tuberculosis in the Ohio study numbered 43 in the vaccinated and 6 in the controls. If the morbidity rate among the vaccinated had been the same as in the controls, 36 cases would be expected in the vaccinated; thus, the morbidity rate in the vaccinated is actually somewhat higher than in the controls. Again the bulk of the cases occurred among the tuberculin reactors, 286 of the total of 335 cases reported so far.

What conclusions can be drawn, now, from all of this work? First,—and all four studies agree on the point—a very large proportion of the tuberculosis which appeared in each population during the first few years after the vaccination program occurred in the group who were not vaccinated because they had already been infected. This implies that mass vaccination, even if BCG is effective, cannot be expected to have a large and immediate influence on tuberculosis mortality and morbidity rates.

The second major point more or less follows. So little tuberculosis has occurred among

those eligible for vaccination, whether or not they were vaccinated, that the number of cases and deaths is still too small to provide any definite evidence on the effectiveness of BCG. It is possible that, in the Indian and Puerto Rican mortality data, there may be early signs that BCG, under certain circumstances, could be useful in tuberculosis control. On the other hand, it is also true that in none of the morbidity comparisons is there any evidence of a beneficial effect of BCG.

The third point is that these studies do not indicate that tuberculosis in this country would be more effectively controlled by adding mass vaccination programs. Muscogee County in Georgia may not be entirely representative of the whole country but, since the effect of the vaccination program there is imperceptible, it seems that there is little reason to expect very different results in other communities.

In the present report there has been time only to describe very briefly the Public Health Service studies. Neither these studies nor those in recent reports by others indicate a need for changing the Public Health Service recommendations of three years ago.

DIVISION OF CHRONIC DISEASE AND TUBERCULOSIS
PUBLIC HEALTH SERVICE
U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
WASHINGTON, D. C.

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July 15, 1953.

Correspondence

ABORTIVE TUBERCULOSIS IN GUINEA PIGS INDUCED BY PATHOLOGIC MATERIAL CONTAINING YOUNG TUBERCLE BACILLI

To the Editor of the American Review of Tuberculosis:

The controversy created during 1922 to 1933 by the publications of Vaudremer, Valtis, Bezançon and Hauduroy, Durand, and Arloing and Dufourt concerning the filterable forms of tubercle bacilli was ended with the acceptance of the opinion that errors had occurred in the course of these studies and that the conclusions drawn by several of the investigators could not be confirmed. These studies have been lost sight of for the past twenty years. As is so often true, the unfavorable reaction to the original studies was exaggerated since in rejecting it the wrong interpretation of the facts was confused with the facts themselves, a point which we have recently discovered to be valid. On the basis of research extending over many years, we wish to separate in this letter the erroneous interpretation from the facts.

Calmette and Valtis in 1926 described a benign form of tuberculosis in guinea pigs inoculated with the filtrate of a culture of tubercle bacilli passed through a Chamberland L3 filter, under a pressure of 25 to 30 cm. of mercury. Under similar conditions these filters do not permit the passage of the ordinary tubercle bacilli. The mild infection induced in these guinea pigs was associated with transitory tuberculin sensitivity and a transitory swelling of certain lymph nodes. Prolonged search of smears from these lymph nodes revealed an occasional clump of either nonacid-fast or more or less acid-fast organisms. Inoculation of these lymph nodes on egg medium and into guinea pigs gave negative results. Lesions presenting the same characteristics were observed by Calmette, Valtis, and Lacomme (1928) in guinea pigs inoculated with the unfiltered mesenteric nodes of an infant born from a tuberculous mother. Similar observations were subsequently made by other investigators following the injection into guinea pigs of blood or urine from tuberculous or suspected tuberculous persons and in guinea pigs inoculated with synovial fluid, hydrocele or ascitic fluid, bone lesions, or cutaneous tuberculous lesions. In view of these findings it was assumed that the elements which passed through the Chamberland L2 or L3 filters were not the usual forms of tubercle bacilli but were smaller forms comparable perhaps to an ultravirus. It was thought that there must be a relationship between the small filterable elements and the atypical tuberculous lesions since the inoculation in guinea pigs of tissues or liquids derived from atypical lesions, as well as filtrates of cultures of tuberculous material, can produce in these animals the Calmette-Valtis type of infection. Other investigators who failed to reproduce these results believed that this type of infection was produced by the inoculation of small numbers of tubercle bacilli or by fragments of dead tubercle bacilli which had passed through the filters and sensitized the animals to tuberculin.

In view of these differences of opinion, we have undertaken to study this question guided by two observations which pointed the same way. One was the demonstration by several authors that filterable forms of tubercle bacilli were demonstrable only in cultures which were less than eight days old. The other noted by one of us (L. N.) in association with A. Boquet and Valtis[1] and confirmed recently[2] was that cultures of tubercle bacilli in their first days of growth, either in the film on the surface of Sauton medium or in the

[1] NEGRÉ, L., BOQUET, A., AND VALTIS, J.: Ann. Inst. Pasteur, 1930, *94*, 247.

[2] NEGRÉ, L.: Ann. Inst. Pasteur, 1947, *73*, 713.
Les Lipoides dans les Bacillies Tuberculeux et la Tuberculose, Masson et Cie, Paris, 1950.

depth of Besredka medium, have a slight sensitizing power and are conspicuously less toxic and virulent than older bacilli.

As a result of numerous experiments,[3] we have drawn the following conclusions:

Under a pressure of 25 to 30 cm. of mercury, tubercle bacilli originating from cultures less than six days old do not pass through a Chamberland L3 filter. Ordinary bacteria do not pass through the filters under these conditions.

Under a pressure exceeding 50 cm. of mercury, bacilli derived from a culture less than six days old often pass through the Chamberland L3 filter. When this filtrate is injected in large quantities subcutaneously into guinea pigs and intravenously into rabbits, the animals frequently become sensitized to tuberculin and in certain cases lesions are induced.

The filtrate obtained under the same high pressure from viable thirty-day-old cultures of tubercle bacilli or from young or old cultures killed by heat fails to induce sensitivity to tuberculin in the treated guinea pigs and rabbits.

One may therefore discard the hypothesis that the elements which pass through the Chamberland filter are extremely minute forms comparable to an ultravirus or that they represent fragments of dead tubercle bacilli and that these particles are capable of sensitizing animals to tuberculin. Only the young forms of tubercle bacilli are able to pass through the Chamberland L3 filter and only under a pressure greater than 50 cm. of mercury. It is probable that the errors which have occurred were due to sudden changes in pressure greater than 30 cm. of mercury which permitted the occasional passage of young bacilli through the filters.

The mild infection, type Calmette-Valtis, is produced in guinea pigs by the inoculation of some component of young tubercle bacilli as we have shown by the production of such a type of tuberculosis in guinea pigs following the injection of unfiltered young cultures.

One of the writers (J. B.)[4] has noted that the inoculation into guinea pigs of a single unit of bacilli originating from young cultures transplanted every three days produces in these animals either a transitory hypersensitivity or progressive lesions. Similarly, the inoculation subcutaneously into guinea pigs or intravenously into rabbits of 1 to 10 units of such organisms is capable of inducing a transitory sensitivity to tuberculin or of producing discrete tuberculous lesions.[5] We have obtained the same results by the inoculation of filtrates of young cultures obtained under a pressure in excess of 50 cm. of mercury.

Because the inoculation into guinea pigs of an occasional young tubercle bacillus produces in certain instances a transitory type of tuberculosis, it was thought that it might be possible to encounter a benign infection in guinea pigs inoculated with pathologic material from tuberculous or suspected tuberculous patients, especially in the early stage of the disease.

Of 405 specimens submitted for examination for tubercle bacilli, 135 have given positive results by guinea pig inoculation and by culture on egg medium. Among the 270 other cases which proved to be negative following guinea pig inoculation and culture in egg medium, we have found 26 cases in which certain of the animals showed a transitory tuberculin sensitivity accompanied by an enlargement of sublumbar and tracheobronchial lymph nodes and occasionally a granular appearance of the spleen. Cultures of the nodes and reinoculation of these nodes into normal guinea pigs were negative as in the case of the lymph nodes of the guinea pigs inoculated with the filtrate of young tubercle bacilli which induced tuberculin hypersensitivity in these animals.

[3] Negré, L., and Bretey, J.: Ann. Inst. Pasteur, 1952, *82*, 1.

[4] Bretey, J.: Rev. de la tuberc., 1943, *8*, 163.

[5] Negré, L., and Bretey, J.: Ann. Inst. Pasteur, 1952, *82*, 132.

In 12 of the 26 cases, the abortive form of tuberculosis developed in guinea pigs following the inoculation of blood from 5 cases of erythema nodosum, from 3 cases of pulmonary tuberculosis, from 3 cases following the injection of blood of hyperthermia, and of blood from the umbilical cord of a tuberculous woman. This form of abortive type of tuberculosis was also elicited by the inoculation of spleen or mesenteric nodes from infants born from tuberculous mothers, from urine, from pleural fluid, from gastric lavage, and following the inoculation of skin lesions and bone lesions.

It was with analogous unfiltered material that Calmette, Valtis, and Lacomme (1926) and later other investigators (1936) obtained by direct inoculation into guinea pigs a benign form of tuberculosis identical with that which we have observed in these animals, following the inoculation of filtrate of young cultures of tubercle bacilli obtained under high pressure or by the inoculation of several units of young unfiltered tubercle bacilli.

It is essential to call attention hereafter to those instances in which guinea pigs inoculated with pathologic material from suspected cases of tuberculosis show a transitory tuberculin hypersensitivity associated with a transitory hypertrophy of certain lymph nodes, especially of the tracheobronchial nodes. Such findings are proof that the pathologic material contains incompletely developed tubercle bacilli.

It has been shown that young tubercle bacilli do not have the same chemical composition which characterize them later. A large number of the organisms are not acid fast. We know, on the other hand, from the recent studies of Asselineau (1951) that the lipid content of young bacilli is approximately one-third that of the fully developed organisms. On the basis of their chemical composition, one can explain the ability of the young tubercle bacilli to pass the Chamberland L3 filter under sufficient pressure, and their slight pathogenicity for guinea pigs. Because of the marked reduction of their lipid content, they are significantly more vulnerable to the defense mechanisms of the organisms than are the fully matured tubercle bacilli. It is quite possible that the fixation of the lipids by the newly formed bacilli results with more or less rapidity, depending on the organ in which they are deposited by the circulating blood.

One may formulate the hypothesis that the newly formed bacilli originate from certain organisms of an exogenous primary infection or from the center of a tuberculous process present in a heavily vascularized organ. In the latter event the organisms are discharged by the bloodstream into the urine or through serous exudate. The presence of these organisms in the center of bone and in the cutaneous lesions can be explained by the fact that, in these tissues, conditions are not sufficiently favorable for them to rapidly develop their complete chemical constitution. Such is probably the reason why strains of tubercle bacilli of low virulence are frequently described in skin lesions. On the other hand, we have never established the abortive type of tuberculosis in guinea pigs inoculated with the sputum of tuberculous patients. This is probably due to the fact that these ulcerating lesions are old or that the tubercle bacilli find the lipid-rich lungs a particularly favorable place for their development. One can assume that the spontaneous healing of certain primary lesions and the more prompt healing of pleurisy, serofibrinous peritonitis, and acute miliary tuberculosis depends in great part on the special characteristics of the immature tubercle bacilli.

In conclusion, we believe that one should discard the idea that certain atypical lesions of tuberculosis are due to an ultravirus of tuberculosis.

The benign tuberculous infection described in guinea pigs inoculated with pathologic material, which gives negative results when planted on egg medium and which fails to produce progressive tuberculosis in inoculated guinea pigs, is an indication that the material contains tubercle bacilli comparable to those present in young cultures.

The fact that human pathologic material has produced this infection in guinea pigs in

half of the cases with samples of blood and in the other half, especially, with urine and serous discharges is an indication that the tubercle bacilli must frequently circulate in the blood in an immature form.

PASTEUR INSTITUTE
PARIS, FRANCE

Sincerely yours,

L. NEGRÉ
J. BRETEY

March 11, 1953

TOXICITY OF ISONIAZID FOR THE MONKEY

To the Editor of the American Review of Tuberculosis:

The very interesting article entitled "The Toxicity of Isoniazid for the Rhesus Monkey" by L. H. Schmidt, R. Hoffmann, and H. B. Hughes, in the AMERICAN REVIEW OF TUBERCULOSIS, Volume 67, June, 1953, page 798, merits comment. The authors recommend systematic trials of considerably higher doses of isoniazid in the human. This is based on their experience which indicated low toxicity of isoniazid in the monkey. Our limited study of monkeys confirms this fact. There is a distinct species difference in the toxicity data when comparing information derived from dog and monkey. However, this is merely one more example, which could be multiplied a great many times, of species variation in toxicity for any compound. It still seems completely impossible to predict accurately the toxicity in man from the toxicity data in either the dog or the monkey. Approximate ranges may be derived, but the toxicity must always be determined directly in the human. In the case of isoniazid, the growing body of experience confirms the impression derived in 1951, namely, that isoniazid is less toxic for laboratory monkeys than for man. The types of toxicity in man are indicated well enough by the experience with dogs. The experience with monkeys is less satisfactory, both qualitatively and quantitatively, as a basis for prediction than the canine data.

This information, assembled from a large number of investigators in a period of two years, indicates a risk of advising general employment of doses of isoniazid in excess of 10 mg. per kg. per day. An exception should be made for very brief periods of intensive treatment, as in meningeal or miliary tuberculosis. When the doses are significantly above 5 mg. per kg. per day, there is reason to believe that the frequency of peripheral neuropathy and central nervous system disturbances will be significantly increased.

I am in agreement with the above-mentioned authors that there are advantages to be obtained from an increased use of monkeys in experimental pharmacology. This is, however, not to indicate that we should abandon the use of the dog, but rather that we should increase the variety of observations as far as possible prior to approaching human therapy.

HOFFMANN-LA ROCHE, INC.
NUTLEY, NEW JERSEY

Sincerely yours,

ELMER L. SEVRINGHAUS

July 14, 1953

PROCEDURE WITH NEGATIVE CULTURES FOR TUBERCLE BACILLI

To the Editor of the American Review of Tuberculosis:

It is my understanding that the Communicable Disease Center of the Federal Security Agency recommends the smearing and staining of the surfaces of all negative cultures for tubercle bacilli in order that positive cultures of bovine or dysgonic human tubercle bacilli will not be overlooked. Therefore, we have been following this plan since our hos-

pital opened in August, 1951. To date, in well over 2,500 negative cultures, we have had no growth of either bovine or dysgonic tubercle bacilli.

Since the above procedure is time consuming and causes a delay in the reporting of results, I have been wondering if we would be justified in discontinuing it. I would appreciate your views on the subject and those of your readers.

OHIO TUBERCULOSIS HOSPITAL
COLUMBUS 10, OHIO

Sincerely yours,

CONSTANCE J. CONNORS

July 14, 1953

Books

FAMINE DISEASE IN GERMAN CONCENTRATION CAMPS. COMPLICATIONS AND SEQUELS WITH SPECIAL REFERENCE TO TUBERCULOSIS, MENTAL DISORDERS, AND SOCIAL CONSEQUENCES. By Helweg-Larsen, Henrik Hoffmeyer, Jorgen Kieler, Eigil Hess Thaysen, Jorn Hess Thaysen, Paul Thygesen, and Munke Hertel Wulff. Pp. 460, Ejnar Munksgaard, Copenhagen, 1952.

The seven Danish physicians who contributed to this book state that one of their objects was to express in medical terms and figures some of the human misery in the Nazi concentration camps during World War II. Most of their personal observations deal with Danish internees, particularly young men of the Resistance Movement and of the Police Force. In reality, however, the theme of the book is broader; it concerns human starvation with all its physical, mental, social, and political consequences. The Nazi concentration camps became during the last War the theatre where was enacted the largest, and most systemically organized, starvation tragedy in the history of mankind. But starvation does not occur only in war time. The authors always have in mind that, in any period, a large part of the world population lives in a state of famine. The achievements of medical science and the activities of philosophers and statesmen will remain in many places of little account until society has learned to meet the most essential need of man, what the authors call his "primary right, the right to have enough to eat."

There are so many fascinating aspects to this book that it is difficult to select from its wealth of factual information: the details of life in the camps, the multiple physiological and psychic facets of famine disease, the mental deterioration which it brought about, the unforeseen and pathetic problems—medical and social—of repatriation. It is worth mentioning in this respect that no adequate knowledge is available for bringing the starved person back to a state of normal nutrition. As the late Sir Jack Drummond wrote a few years ago, "Although in our lifetimes, millions have died from starvation, . . . it is not possible to find clear-cut advice how to resuscitate people who are near death from this cause. That fact is, I think, a terrible reflexion on our lack of concern for the human race, as a whole."

As poorly understood, and as interesting, as the pathological manifestations of famine is the fact that certain diseases (or symptoms of disease) were extraordinarily rare, or much decreased in severity, while the men remained in the concentration camps—for example, dental caries, peptic ulcer, appendicitis, cardiovascular disorders, diabetes, thyrotoxicosis, rheumatic fever, glomerulonephritis, and allergic conditions in general.

It is not possible to consider in this review the extensive descriptions given by the authors of the various manifestations of famine disease (hunger cachexia, hunger edema, polyuria, hunger diarrhea, alimentary diarrhea, cardiovascular disorders, disorders of the blood, of metabolism, and of the endocrine system, avitaminosis, mental deterioration). Nor shall we outline the very difficult scientific and practical problems (medical and social) presented by the repatriation of the internees. Instead, we shall have to limit our remarks to the field of infectious diseases, and especially of tuberculosis.

The epidemiology of infection among the internees was peculiar in several respects. On the whole, acute epidemics were rare, the most common medical problems being caused by microbial agents endemic in western Europe.

When an epidemic disease was introduced into a camp (e.g., typhus in the Dachau camp) it raged with extreme violence, but generally speaking the morbidity in

the camps consisted mainly in an increase in infectious diseases which are nowadays normally endemic in the European communities. The most common infectious disorders were: 1) respiratory tract infections including cold and bronchopneumonia, 2) skin infections (folliculitis, infected ulcers of the crura, etc.) which might give rise to diffuse, but usually torpid, cellulitis; and 3) pulmonary tuberculosis.

A survey of the present material seems to indicate that the increased incidence of pneumonia, skin infections, and tuberculosis bears a definite relation to the nutritonal condition.

All reports indicate that the tubercle bacillus was probably the infectious agent most commonly contributing to death during deportation. The tuberculosis mortality reached a high of 40 per cent of the total mortality at Buchenwald and 30 per cent at Porta, two of the worst camps. The occurrence and course of tuberculosis was studied in great detail among the Danish prisoners, a group of particular interest since it consisted largely of young robust persons in whom the disease was extremely rare before deportation.

The authors have made a particularly careful study of the relation of tuberculosis morbidity to the state of tuberculin allergy. They summarize their findings in the accompanying table:

FREQUENCY OF TUBERCULOSIS IN THE VARIOUS IMMUNITY GROUPS

TUBERCULIN REACTION BEFORE DEPORTATION		TUBERCULIN REACTION AFTER REPATRIATION									
		Pulmonary Tuberculosis		Extra-pulmonary Tuberculosis		Primary Pleurisy		Pulmonary Infiltration		Nothing Abnormal	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Tuberculin-negative	59	9	15	4	7	3	5	4	7	39	66
BCG vaccination	57	5	9	0	0	2	4	5	9	45	78
Tuberculin-positive	316	9	3	0	0	20	6	9	3	278	88
Reaction unknown	820	28	3.4	1	0.1	28	3.4	17	2.1	746	91

and in the following statement:

In our total case material pulmonary tuberculosis was suspected in 9 per cent of the persons who had received BCG . . . . Thus, the frequency of pulmonary tuberculosis occupies an intermediate position between those demonstrated in 'tuberculin-negatives before deportation' (15 per cent) and 'tuberculin-positives before deportation' (3 per cent). This seems to indicate that BCG vaccination has to some extent been capable of preventing the occurrence of tuberculosis during the deportation.

It is true that the frequency of tuberculosis among the persons vaccinated with BCG was essentially higher than among the primarily tuberculin-positive reactors, but a direct comparison is scarcely quite permissible. The group 'tuberculin-positives before deportation' at least represents a selected material, since all those in whom the primary tuberculous infection caused disease had been excluded. Accordingly, the difference in the frequency of tuberculosis in the two groups may not be quite as large as the figures seem to indicate.

As may be seen in the table, pulmonary infiltrations were at least as frequent in persons vaccinated with BCG before deportation as in the controls, but the authors are careful to point out that these infiltrations may not have been of tuberculous etiology since the

presence of tubercle bacilli could not be demonstrated. It was found, on the other hand, that in the BCG-vaccinated group as a whole, the sensitivity to tuberculin after repatriation was appreciably higher than that observed as a result of vaccination—a fact which "presumably indicates that these vaccinated persons had been exposed to tuberculous infection." It seems pertinent to note that mere exposure could hardly bring about an increase in allergy; this could occur only if actual bacterial multiplication had taken place, either the result of superinfection with virulent bacilli or of reactivation of the BCG organisms in bodies weakened by the abnormally unfavorable living conditions.

Tuberculosis presented some interesting and puzzling clinical features among the men during and after deportation. The disease followed a rather torpid course in the undernourished patients, whereas a flare-up was often observed when refeeding was begun. This observation is explained as follows by the authors:

> As is well known, the tuberculin allergy may be reduced or even disappear in severe cachetic cases of tuberculosis. A possible decrease in allergy will result in a reduction of the cellular reaction which on X-ray examination is the most conspicuous sign of the tuberculous process. If this view is correct, the flare-up of the tuberculosis during realimentation is only apparent and an expression of the regenerated allergy and subsequent increased cellular reactivity.

Surprisingly enough, one-third of the cases of tuberculosis did not become manifest until two to four years after repatriation, although all Danish prisoners returned to good sanitary and nutritional conditions. On the other hand, the prognosis for all the Danish tuberculous patients under study was far better than average.

> During the five-year observation period only 5 deaths due to tuberculosis occurred among the 62 unquestionable active cases of tuberculosis. This low mortality has also been observed by . . . other writers. As the patients were treated prior to the introduction of streptomycin, most of them have been cured by ordinary sanatorium treatment, by which the general reduction of their resistance which had occurred during their stay in the camps has been overcome.

After repatriation following a relatively short period of internment (of the order of six months), it was found that the frequency of tuberculosis among the members of the Resistance Movement and of the Police Force was respectively "12 and 5 times as high as among the corresponding age groups of the male population of Copenhagen within the same period." The differences observed between the Resistance Movement group and the Police Force may have been due, in part, to the fact that the latter enjoyed a better diet through a regular supply of Red Cross parcels, even though both groups were interned in camps of the same type where conditions of exposure were the same. Most of the Police Force prisoners, for example, were interned at the notorious Buchenwald camp. In general, it appeared as if the morbidity from tuberculosis depended more on the conditions during internment (nutritional deficiency and its attendant weight loss) than on the duration of deportation.

> However, from this it must not be concluded that starvation with its attendant reduction in resistance to infection was the only factor at work in the production of tuberculosis, since usually camps with the greatest degrees of dietary insufficiency also otherwise provided the poorest sanitary conditions of life. It will, therefore, be very difficult to differentiate between the share due to the exposure to infection and that due to the reduced resistance to infection.

Granted these wise reservations, the facts reported leave little doubt that starvation was an important contributing factor to the severity of tuberculosis in the concentration camps. There have been published, of course, many epidemiological studies in support of the widely held clinical view that malnutrition increases susceptibility to tuberculous infection, but all these studies deal with highly complex situations difficult of analysis. The case of the Danish prisoners has convincing value, dealing as it does with robust young men, removed from a country with high nutritional standards and extremely low tuberculosis rates, who developed severe disease when exposed under conditions of semi-starvation and overcame it (without the help of chemotherapy) shortly after their return to a favorable environment. Moreover, as already noted, tuberculosis among them was less prevalent in the group (the Police Force) whose diet was less drastically reduced. It is worth noting that the observations made during the war, taken together with the results of studies in experimental animals, suggest that it is only when malnutrition reaches an extreme degree that it is accompanied by an increased susceptibility to tuberculosis. This fact suggests that metabolic disturbances rather than specific nutritional deficiencies constitute the real link between nutrition and disease.

It is impossible to convey in a review the magnificent quality of scientific workmanship, and the depth and breadth of understanding, that went into the making of this book. Furthermore, the authors have brought to it more than medical knowledge and wisdom. They have carved their subject in the form of a social document, made out of the worst and the best in human behavior. Four of the authors were deported as participants in the Resistance Movement, and they experienced at first hand the horrors of concentration camps. Yet, only concern for the dignity of man, and never personal rancor, gives emotional color to their factual descriptions. In the midst of unheard of misery and sufferings, they succeeded in making an objective study of disease while serving as helpful physicians whenever opportunity presented itself. Their performance constitutes more than an important contribution to medicine; it is also a symbol of the hope that, despite the evidence of so much bestiality remaining in the world, man need not despair of mankind.

René J. Dubos
New York, New York

Tubercle Bacillus and Laboratory Methods in Tuberculosis. By M. A. Soltys, C. A. St. Hill, and I. Ansell. Pp. 212, E. and S. Livingstone, Ltd., Edinburgh and London, 1952, Price, $4.50.

According to the authors, "This book is an attempt to review the vast literature on the tubercle bacillus in an objective manner and to give practical details of laboratory methods in tuberculosis." The result falls somewhat short of this goal, mainly because of the impossibility of dealing adequately with the bacteriology and immunology of tuberculosis in the small space allotted to this subject in Part One (66 pages). In spite of the incomplete coverage of the literature and inadequate discussion and analysis, the choice of references for this section is good and the major emphasis is on recent work. As a very brief review of some of the more important aspects of our knowledge of the *Mycobacteria*, this section has considerable merit.

Part Two, which is concerned with laboratory methods in tuberculosis, is more extensive and covers adequately the laboratory diagnostic procedures, the cultivation of the *Mycobacteria*, and the inoculation of experimental animals. There is also a brief discussion of the experimental chemotherapy of tuberculosis and the nature and uses of tuber-

culin. On the whole, this section is well done and should serve as a useful guide to the laboratory worker.

GUY P. YOUMANS
Chicago, Illinois

BCG VACCINATION. Studies by the WHO Tuberculosis Research Office, Copenhagen. By Lydia B. Edwards and Carroll E. Palmer, with the assistance of Knut Magnus. Pp. 307, World Health Organization, Palais des Nations, Geneva, 1953, Price, $3.00.

This monograph contains a series of reports on the study of BCG Vaccination by the WHO Tuberculosis Research Office during the period November, 1949, to September, 1952. The research was done in connection with the international BCG vaccination campaigns among school children in four different countries. Various aspects of BCG vaccination were studied, including the effects of long-continued storage, heat, light, changes in the technique of intracutaneous vaccination, variation in the preparation and concentration of the vaccine, mixtures of living and dead bacilli in varying proportions, and the variability of vaccines prepared by different production centers. Practically all of these studies have been previously reported in various medical journals, and are already well known to workers in this field.

There is no need to review the results of these studies or the inferences the authors make from their observations since the volume is readily available. However, it is probably important to emphasize that these studies have a significance beyond their specific results with regard to BCG vaccination. First of all, they indicate how much important fundamental research can be accomplished within the framework of a service program; this is a research potential which has too frequently been disregarded. Secondly, it points up the paradoxical situation where our ignorance of the basic biological actions of a vaccine far exceeds our knowledge, despite the fact that the agent has been used in various parts of the world for several decades. Last of all, it indicates how fruitful a quantitative approach can be when properly applied to observations in human populations.

The authors are to be commended on their presentation in the appendix of all the basic data upon which their analyses and inferences are based.

ABRAHAM M. LILIENFELD
Baltimore, Maryland

BOOKS RECEIVED

SECTIONAL RADIOGRAPHY OF THE CHEST. By Irving J. Kane. Pp. 154, with illustrations, Springer Publishing Company, Inc., New York, 1953, Price, $7.50.

ANNUAL REVIEW OF MEDICINE. Volume 4. Pp. 452, Annual Review, Inc., Stanford, California, 1953, Price, $6.00.

THE METABOLISM OF THE TUBERCLE BACILLUS. By William F. Drea and Anatole Andrejew, with a foreword by Esmond R. Long. Pp. 448, Charles C Thomas, Springfield, Illinois, 1953, Price, $12.50.

CLINICAL DIAGNOSIS BY LABORATORY METHODS. Twelfth Edition. By James Campbell Todd, Arthur Hawley Sanford, and Benjamin B. Wells. Pp. 998, with illustrations, W. B. Saunders Company, Philadelphia and London, 1953, Price, $8.50.

American Trudeau Society

Medical Section of the National Tuberculosis Association

Report of Panel Discussion on Survival and Revival of Tubercle Bacilli in Healed Tuberculous Lesions

The following represents the transcript of the panel session entitled "Survival and Revival of the Tubercle Bacillus in the Apparently Healed Tuberculous Lesions" held on May 18, 1953, at the annual meeting of the National Tuberculosis Association in Los Angeles, California.

The only editorial changes made have been minor ones, made solely in an effort to improve readability.

Dr. Gladys L. Hobby acted as moderator.

DR. HOBBY: Our panel discussion today is on the survival and revival of tubercle bacilli in the healed tuberculous lesion. Our basic problem is: If a tuberculous lesion contains acid-fast bacilli which are visible on smear but no organisms can be recovered by cultivation on artificial media or by guinea pig inoculation, is one justified in concluding that the organisms are dead? That is our basic question for discussion today and, needless to say, this will involve discussion also of the evidence which is available now and the evidence which should be obtained in order that we may have a definitive answer to this question.

Before we proceed, I would like to introduce to you our panel members and then we will go ahead in a very informal fashion and see what information we can obtain on this subject.

At the far end of the table, at my left, Dr. Nicholas D'Esopo, Chief of Tuberculosis, West Haven Veterans Administration Hospital, West Haven, Connecticut; Dr. David Smith, Professor of Bacteriology and Associate Professor of Medicine, Duke University School of Medicine, Durham, North Carolina; on my left, Dr. Walsh McDermott, Associate Professor of Medicine at the New York Hospital-Cornell University Medical Center, New York City, and Editor of THE AMERICAN REVIEW OF TUBERCULOSIS. On my immediate right, Dr. William H. Feldman, Mayo Foundation, Rochester, Minnesota; Dr. Sidney Raffel, Professor of Bacteriology and Experimental Pathology, Stanford University School of Medicine, Stanford, California; Dr. Oscar Auerbach, Pathologist, East Orange Veterans Administration Hospital, East Orange, New Jersey; and at the extreme end, Mr. William Steenken, Jr., Director of the Trudeau Laboratory, Trudeau, New York.

We will now go ahead being very informal, and I think that we might suggest that the panel members use first names only so as to avoid confusion. We won't remember to be formal so there is no use trying. Please bear in mind that, if we speak directly into the microphone, it will be easier for the audience and for those who are trying to get an informal record of this discussion.

I think we might start our panel discussion by trying to orient ourselves a little bit as to what has been done up to the present time,—what the present situation is. Nick, if I understand it correctly, the study of the bacteriology of the resected lesion was first started at the Sunmount VA Hospital. Can you tell us very briefly how you came to undertake this study?

DR. D'ESOPO: Our study started in 1948, in the fall of 1948; it was primarily a bacteriological study in which we tried to answer the question: Does PAS when given with streptomycin over a prolonged period significantly alter the sensitivity of bacilli to streptomycin. It was a long-term study of streptomycin combined with PAS. We soon acquired a group of patients who had come to the point where cavities were closed, the cultures were persistently negative, and X-rays were stable. A great majority of these patients had negative cultures, so we were actually thwarted in our original study of the bacteriology of the organisms in the sputum, that is, of the sensitivity of those organisms. It was at this time that Dr. Medlar, who was then our pathologist, suggested that these lesions be resected. He thought they were necrotic residual foci and should be resected. This we did, and he was entirely correct; these lesions were necrotic; many of them were filled-in cavities; and most of them contained large numbers of bacilli which could be stained and which looked perfectly normal. These tubercle bacilli were first cultured for the purpose of determining their streptomycin sensitivity. We have not been able to determine their sensitivity because the cultures were negative. Much to our surprise, the bacilli did not grow out. In some cases the numbers of bacilli were very great. This was actually the beginning of the study and from then on it was a question of selecting the numbers of cases and actually running the bacteriological studies. In brief, one might say that the only real correlation between nonviability and the lesions is the closure of cavities; if the cavity has closed, it is difficult to grow the bacilli in the great majority of cases.

DR. HOBBY: Then, this is really the outgrowth of the long-term treatment of patients with streptomycin and PAS, is it not?

DR. D'ESOPO: Yes, it all started with the attempt to determine how PAS affected the sensitivity of organisms over prolonged periods of time.

DR. HOBBY: How many lesions have you had a chance to study, Nick?

DR. D'ESOPO: About 175.

DR. HOBBY: And your percentage of positive cultures is about what?

DR. D'ESOPO: Well, we found that when patients are treated up to 8 months, they yield about 10 per cent, that is, about 10 per cent of these lesions have viable bacilli, emphasizing again the criteria of resection—those cavities with persistently negative cultures and stable X-rays.

DR. HOBBY: About 10 per cent are positive by culture, and how many by smear?

DR. D'ESOPO: About 75 per cent. After the eighth month, if the case is treated with daily streptomycin and daily PAS, we did not have a single lesion yielding viable bacilli. In the cases receiving streptomycin twice a week, we have had some after the eighth month.

DR. HOBBY: Are these the lesions, Nick, which you normally or frequently speak of as the "target point lesions"? What do you mean by that term?

DR. D'ESOPO: These lesions were resected at a point which we define as the "target point," for want of something better; a point at which the cavities are closed, the cultures have been negative for several months, and X-rays have been stable. It is at this point that the lesions are resected and it is at this point that we are trying to make a correlation between the bacteriology and the pathology.

DR. HOBBY: Oscar, can you give us briefly a little picture of the pathology of these lesions of which we are speaking?

DR. AUERBACH: I think the lesions which the pathologist now sees in the closed cavity to which Nick just referred are quite different from what we used to see in the pre-chemotherapy days. In the pre-chemotherapy days, the cavity closed, the bronchial

outlet became obliterated, the contents became inspissated, and, by and large, that was the end of that picture. The calcium would be deposited and the cavity walls would become hyalinized. In the long-term chemotherapy cases, we see that the cavity wall closes; the bronchus leading into the cavity undergoes re-epithelialization. The lumen of the cavity diminishes in size. There is healing of the cavity wall but, in contrast to the pre-chemotherapy days, there is now a bronchial outlet. We have also seen cases under prolonged chemotherapy in which, even after the contents became calcified, late liquefaction occurred and evacuation of the contents was observed. So that, in the main, the difference is that cavity closure is present in both instances but now there is a bronchial outlet leading to the cavity.

DR. HOBBY: Am I correct, Nick, that all of your cases are patients who have had chemotherapy?

DR. D'ESOPO: No, we have had a few people who, principally for diagnostic reasons, were resected without chemotherapy—older people in whom a diagnosis of carcinoma had been obtained—and we have several where bacilli were also nonviable, so that we have said that the nonviability of tubercle bacilli is not directly related to chemotherapy.

DR. HOBBY: Now, before we go any further in our discussion, I think it might be of interest to make a few statements about the work which has been done by others who are not represented on our panel. At the VA meetings last February, there were six hospital groups, including Dr. D'Esopo's, who reported on the bacteriology of the resected lesion. I have tried to tabulate the data from these six hospitals and, in doing so, we must recognize the fact that not everyone has described the lesions in the same terminology, not everyone has described the bacteriology in full detail, so this may not be an exactly accurate statement of the picture, but it is a general statement anyway. In the first place, 515 resections were reported by the six hospitals. Bacteriological studies were conducted on 661 apparently closed cavities. Of these, more than 346, or approximately 52 per cent, were reported as positive for acid-fast bacilli by smear. The remaining patients were not reported as to what the smears showed. Only 85 of these, or 12.8 per cent, were reported as positive for acid-fast bacilli by culture and/or guinea pig inoculation. Now there are only 23 apparently open cavities which were described. Of these, 70 per cent were reported positive by smear and 74 per cent by culture or guinea pig inoculation. So we have there, I think, abundant confirmation of what Nick has been saying so far; abundant confirmation that, at the present time, with the technical methods used so far, there is this type of lesion which shows organisms on smear but from which one cannot cultivate them.

I think it would be of interest to examine briefly, if we can, what the situation is in those patients who have not had chemotherapy. Bill Feldman, do you have some information on that?

DR. FELDMAN: This problem which has become so terribly important in the last few years is by no means a new problem. As a matter of fact, for more than fifty or seventy-five years it has been observed by careful workers that, in a considerable percentage of all lesions in which there was retrogression, there were tubercle bacilli or so-called tubercle bacilli, these organisms being bacillary forms which one could not culture and which were unable to produce progressive tuberculosis in a susceptible animal such as a guinea pig. I recall particularly the really comprehensive study of A. Stanley Griffith published in 1929, something over 300 cases, which entailed this sort of study. I remember this was in the pre-chemotherapeutic era. None of these patients received what we now consider to be specific chemotherapy. Of the something over 300 cases, there were 176 specimens in which they were able to demonstrate the presence of these acid-fast

bacillary forms, but were unable to produce any sort of what we recognized to be tuberculous disease or guinea pig lesions. About 97 of these tissues, if I remember correctly, were negative by culture. So you see it is not by any means a new problem.

DR. HOBBY: I understand that none of us have been talking loud enough for the microphone so we will all have to speak up a bit.

Is there other information or do any of the rest of you have information on patients who did not have chemotherapy?

DR. FELDMAN: I'd like to finish; just another short statement. Some eight or ten years ago we studied a series of 29 children who died of causes other than tuberculosis; their ages were from about three months up to fifteen years. We were interested in the potential residual infectivity of the primary Gohn complex. None of these patients, as you can imagine, had any chemotherapy; in fact, that was before the era of chemotherapy. Of these 29 patients there were only 4 in whom we were able to demonstrate living tubercle bacilli which produced tuberculosis in guinea pigs. Despite the fact that all this material was also cultured, in only 4 were organisms present which were actually capable of producing tuberculosis in the guinea pigs. In 3 cases in which the caseous material showed microscopically typical acid-fast organisms, none of these 3 produced any evidence of tuberculosis in guinea pigs. These of course were in the group other than the 4; they were in the 25 in which we were unable to show the presence of living tubercle bacilli. This is another example, then, of the fact that this is not by any means a new problem.

We have another series of 66 cases, adults showing again the primary complex, and there was no evidence that these patients had received any specific treatment at all. In only one of the 66 cases could we demonstrate living tubercle bacilli by guinea pig inoculation. Thank you.

DR. HOBBY: Is there other evidence of this?

MR. STEENKEN: May I ask Dr. Feldman a question? At that time did you consider those organisms which you saw by smear, but with which you could not produce disease or growth; did you consider them dead?

DR. FELDMAN: Well, to me they looked like any corpse; one wouldn't know.

DR. HOBBY: Bill, you're asking for the answers before we get all the proof.

MR. STEENKEN: I don't want the answer. I just want a commitment because it will help in the end.

DR. MCDERMOTT: Gladys, may I make a comment? Bill Feldman has made a point that this is a problem of some duration. I would also like to make the point that it is not a problem unique to the tubercle bacillus. I am sure that everybody realizes that, but I think that the point should be made. Ever since the introduction of powerful antimicrobial agents we've all had the experience of patients, with pneumococcal pneumonia or what have you, who have had some antimicrobial therapy before cultures have been taken, presenting a situation in which one can see structures which resemble microbes on stained smear, but in which the exudates do not give organisms upon culture. For example, many of Dr. Tillett's pneumococcal empyemas in the streptodornase studies yielded stained pneumococcus-like organisms with sterile cultures. It is a problem which runs through the whole breadth of antimicrobial therapy and is not unique to tuberculosis.

DR. HOBBY: Are there any other comments that any of you would like to make? I believe that Dr. Yegian has reported a few cases recently. Dr. Katz from the Brooklyn VA Hospital has also, and I believe that they have presented evidence which fits in with what has just been said. So there seems to be an abundance of evidence that these lesions do exist both in the drug-treated and the nondrug-treated cases under suitable conditions. Do you agree with that Oscar?

DR. AUERBACH: Yes.

DR. HOBBY: I think we might, with that much background about the type of lesions and the situation that exists today, turn to the microbiological aspect. Needless to say, the minute you find that you can see organisms but cannot make them grow, the question arises whether they are really dead or whether we just don't know how to handle them; and certainly, by all the evidence available at the present time, one would assume that they might very well be dead, but it is certainly very interesting and stimulating for us to examine this situation. I think we might start doing so by, first of all, outlining what has been done from the microbiological standpoint up to the present time. Since Bill Steenken has had most experience in this regard, I believe we will start with a few questions in that direction. Bill, how many lesions have you had a chance to study?

MR. STEENKEN: Well, I have been associated with Dr. D'Esopo in his study from the start, mostly from the bacteriologic side, and we at Trudeau have studied lesions from Sunmount, Trudeau, and from Saranac Lake and I have studied over a hundred lesions so far. Of course, these lesions have been of all types. Those that were "non-target point" lesions and those that were "target point" lesions.

DR. HOBBY: What part of the lesion have you selected, Bill, for study, the central necrotic portion, or the wall of the cavity, or both, or just what?

MR. STEENKEN: Well, I think most of you know that I wasn't satisfied with the pathologist taking what he wanted and then passing on the rest to the bacteriologist, I felt that we knew enough about the pathology at present and since the attack is mostly upon the etiologic agent, the tubercle bacillus, we therefore have studied the whole lesion whenever the pathologist would let us have it.

DR. HOBBY: When you have had the opportunity to study these lesions, in that fashion, about how many organisms have you seen on smear? Are they numerous, or sparse, or just what?

MR. STEENKEN: Well, they vary. In many of them you can't find any organisms, and in some you find a few, and in others you find myriads of acid-fast organisms.

DR. HOBBY: By myriads, what do you mean? Do you mean 2 or 3 in a field, 100 per field? Or what?

MR. STEENKEN: No, I mean the old Gaffky scale, so many that they are uncountable.

DR. HOBBY: Okay. Now in handling your lesion, Bill, from then on are you homogenizing or digesting? What is the situation?

MR. STEENKEN: The situation, as you all know, in trying to recover tubercle bacilli from any type of diagnostic material is to subject it to the least amount of chemical digestion or treatment that is possible. We know that secondary organisms are practically nonoccurring in most of these solitary tuberculous lesions and filled-in cavities which become inspissated, so all of these lesions are cultured and inoculated directly into guinea pigs. In many instances we have also subjected the same material to culture and guinea pig inoculation after digestion with the minimum amount of sodium hydroxide.

DR. HOBBY: Are these suspensions of digested tissue washed in any way?

MR. STEENKEN: Well, the point has come up recently that, if we would treat these lesions with albumin, we would be able to neutralize the fatty acids, as Dr. Dubos has shown. In our early work we were trying to culture all of these organisms in the Tween-albumin medium. However, the incidence of positive growth there wasn't even as great as on solid media. More recently, it has been stated that, if we had added albumin in higher concentration, we probably could have revived many of these organisms which we hadn't been able to revive otherwise. We have taken many of these so-called "target point" lesions and treated them with albumin and then cultured and guinea-pigged them

and to date we have not been able to "revive" any of these dead organisms with this added procedure.

DR. HOBBY: What media have you used, Bill?

MR. STEENKEN: Well, we have used the Tween albumin medium, Proskauer and Beck's synthetic medium, and blood agar media as well as media with silica incorporated in it. We have tried, of course, the well-recommended media which have been evaluated over a long period of time, such as the ATS medium.

DR. HOBBY: So, in other words, you have tried quite extensively all of the various standard techniques that are usually accepted as suitable for cultivation of the tubercle bacillus. Is that correct?

MR. STEENKEN: Yes.

DR. HOBBY: And have failed to do so, so far, in a significant percentage of instances. Well, now the question, of course, comes up, as we said before, what does this mean? Here we think we have perfectly good cultural techniques and yet they fail to allow for growth of organisms which can be demonstrated by smear. Bill, you would say that these are perfectly typical organisms so far as you can tell morphologically?

MR. STEENKEN: Yes, they are typical and, of course, it brings up the point right now. I believe that the organisms survive in an intact stainable state but the revival of them has not been accomplished and is still questionable in my mind.

DR. MCDERMOTT: I'm sorry, I didn't hear that Bill,—you say you believe the organisms revive.

MR. STEENKEN: They survive.

DR. MCDERMOTT: Survive.

MR. STEENKEN: Don't get me on the loose there. We set up an experiment as soon as we found out that we could see stainable organisms and still could not culture them or produce disease, an experiment to answer the point of how long organisms could remain stainable in the body and still not be culturable. We made a suspension of tubercle bacilli which we killed by heat, as well as one of the H37Ra strain, which is an avirulent living organism which will only produce local nonprogressive disease. We found that by inoculating these suspensions intratesticularly and subcutaneously we could produce local caseous lesions and that after one year and a half we have been able to remove these caseous lesions and still find myriads of acid-fast organisms in the lesions in which we had inoculated the dead bacilli as well as in those animals which were inoculated with living avirulent bacilli. As time went on the incidence of growth or the number of tubercle bacilli which we could grow from the lesions of the guinea pigs which were inoculated with the living avirulent bacilli has become progressively less until at present we are only growing an occasional colony but still on smear we see many stainable acid-fast organisms.

DR. HOBBY: I'd like to go back to one point which we mentioned very briefly a minute ago, namely, the site of the lesion which is taken for culture. The reason I go back to this is that in my own very limited experience, and it is extremely limited in contrast to Bill's, we have felt that there was a considerable difference in the number of stainable organisms, depending on whether you were trying to stain them in the necrotic portion of the lesion or in the wall of the lesion. Oscar, would you like to comment on that a bit?

DR. AUERBACH: I think it's a well-known fact that, when you have a cavity wall, there are soft yellow areas attached to the inner lining and that, if you were to make a smear of this material, it would almost certainly seem like a pure culture of tubercle bacilli, and this material removed and inoculated into guinea pigs would produce very extensive lesions in these animals. I'm talking now of open cavities, so that the material will be—or the bacilli will be—present in the soft yellow zones along the inner surface of the cavity.

DR. HOBBY: Walsh, am I correct that you or some members of your department have been studying the difference in various portions of the lesion and in the viability of the cells?

DR. MCDERMOTT: We haven't done any work specifically on that point, Gladys. Canetti of course did very extensive studies which support your statement, namely, that the particular area of the lesion makes all the difference in the world as to the number of bacilli. Bill Steenken has made the point that in his observation he uses the whole lesion. I would like to ask Bill a question, if I might, and that is, has he done experiments with heat-killed tubercle bacilli in which he did not add the R1 strain; in other words, in which he just dealt with the single culture?

MR. STEENKEN: Walsh, I didn't want to get you mixed up there; but what I meant was that we took two different series of guinea pigs. For one we used the dead bacilli and for the other we used the living attenuated ones. The reason we did that was because we thought we would be left open to criticism in that when we killed an organism by heat, we would coagulate the organism to such a state that the autolytic enzymes or other enzymes would not have been able to break down the tubercle bacilli or their acid-fast property.

DR. MCDERMOTT: I'm still not clear, Bill,—but I don't want to belabor this point—but have you evidence that tubercle bacilli killed by very rough handling but ones which have maintained their integrity as acid-fast rods,—have you evidence that such acid-fast rods can persist for a long period in the tissues of a mammalian host?

MR. STEENKEN: The only proof that I have is by animal experimentation. We have not put them back into humans to find out whether they will survive there. These organisms we know, as far as the guinea pig experiments go and as far as rabbit experiments go—do you want me to talk about that now, Gladys, or later. We have tried to set up types of tuberculosis identical to what you see in human beings by infecting rabbits by inhalation. We have a few of these rabbits with chronic cavitary tuberculosis treated for a year in which we have brought about the resolution and organization of disease. We have removed those rabbits from chemotherapeutic treatment, and after six months to a year we have not seen any relapse of disease in these rabbits, although by X-ray you can see these well-localized caseous lesions. We have killed off many of these rabbits and we have taken each one of the lesions separately, as many as 8 lesions in any one rabbit, and have inoculated each one of those lesions into many guinea pigs, and we have cultured them on various types of media; they contain many bacilli (the bacilli vary from the lesions but some of them contain many bacilli) and we have not been able to grow these organisms or produce disease. We have always been criticized and many say that, if we put these organisms back into the human body or a nonsensitized host, probably they would produce disease in the humans. So, in order to meet this challenge, we have had to create our own environment, that is, we are working with the rabbit, we use the bovine organism, and we have been able to duplicate the situation experimentally and know what we are dealing with from day to day. We know when the lesion starts, we know when we take it out, and we find the same thing, namely, that we cannot grow these organisms or produce disease in the same identical host.

DR. HOBBY: Bill F., do you have anything you'd like to add at this point?

DR. FELDMAN: No, I think we've got plenty of smog at the moment.

DR. HOBBY: I'm beginning to wonder, in view of Walsh's question, if we really know how to kill bacteria or if we ever kill them.

I think we might go on now and think about some of the possible—and I say possible advisedly, as we have no reason for criticizing present techniques—but some of the possible loopholes in the procedures which we have used so far. Bill Steenken has spoken

in terms of digesting tissue with sodium hydroxide. Some of us are using Waring Blendors or homogenizers. I wonder what the relative merits of these techniques may be? Dave, in relation to the work that you have done on the metabolism of the tubercle bacillus, would you have any thoughts concerning the effects of traces of metals or of the quantities of sodium hydroxide used on these cells?

DR. SMITH: Well, it's been known for a long time that sodium hydroxide does kill or render non-cultivatable a considerable number of the bacilli that are present in ordinary sputum cases. Dr. Dubos really raised the question at the meeting down in Atlanta that either acid, or alkalis, or peptones, or other things which are present in the caseous material may be adsorbed onto the surface of the tubercle bacillus in such a manner that the organism will not grow. That is why we all were calling these dead bacilli until Dr. Dubos threw this one in and we had to admit that we couldn't prove that they were dead. All we were proving was that they weren't growing. Now, it would seem as if it should be a very simple matter to see if these organisms have a normal metabolism. You see them in large numbers, if you put them in a Warburg apparatus—somebody who knew how to run it. I don't, but Dr. Pope down in our laboratory does, and has been measuring day after day metabolic effects with known cultures of tubercle bacilli. Well, when I discussed the problem with her, to my disappointment I got the information that the small number (they are many as they appear by the microscope) but the small numbers present in the lesions are entirely too few to be used in Warburg studies to learn about their metabolism. So it doesn't look hopeful from that point of view. I might add that perhaps Dr. Buddingh's new technique of growing tubercle bacilli in egg yolk, as was developed down in New Orleans, should be tried by somebody as another eliminative test. He's done about 150 cases now, comparing cultures and guinea pigs and cultivation in the egg yolk, and they correspond exactly; he gets no false positives and the amazing thing is that in four to six days—always in less than 10 days— you can get a good growth in the egg yolk of the living embryo. Now that would give one more environment in which a sick tubercle bacillus might recover and be moved out of the class of dead ones.

DR. HOBBY: Would you like to elaborate a little bit more, for the benefit of those who are not familiar with Dr. Dubos' work, on what these various substances are which he has spoken of as inhibitors?

DR. SMITH: I couldn't follow him beyond a certain point, I got lost.

DR. MCDERMOTT: Well, one of them is lactic acid, for example, which is present in all inflammatory tissues and which is incredibly active against tubercle bacilli in very minute amounts.

DR. HOBBY: As I understand the situation, Dr. Dubos feels that there are various organic acids, including lactic acid, which accumulate in and around the tuberculous lesion as a result of the anaerobic metabolism of the inflammatory cell, and also perhaps as a result of the activity of certain lipolytic enzymes which he believes are released during necrosis. He has also, as I understand it, demonstrated that there is a basic polypeptide which is present in some tissues which may or does inhibit growth of the tubercle bacillus. Of course we are all familiar with lysozyme which is active in concentration of around 1:100,000—a very small concentration—and at the same time never actually kills the cells. And then of course the work which has been done primarily, I believe, by Dr. Hirsch in association with Dr. Dubos on spermine and spermidine, both of which become inhibitory to the tubercle bacillus after being modified by an enzyme which exists in the tissues and is known as spermine oxidase. These, I think, are some of the factors which Dr. Dubos has been particularly conscious of and has felt may interfere with our cultivation of these organisms. Sidney, would you like to add anything to that?

DR. RAFFEL: Unfortunately, this is the first time I've thought of anything to say, and it may create a little bit of dissention here in the Committee. I don't think that lactic acid, according to either Dr. Dubos or other persons who have worked with it, is very inhibitory for tubercle bacilli in minute amounts. In fact, it is inhibitory in very large amounts only—about 0.45 per cent—an amount which, so far as I know, has never been found to accumulate in any tissues. I think, though, that a major factor here, as Dubos has pointed out, is that small amounts of lactic acid do become effective as inhibitory agents when the oxygen tension is very low. Of course, that now suggestively brings us closer to what might happen in closed lesions. As a matter of fact, Loebel, Richardson and Shors and others—and currently we also during the past couple of years—have found that without any organic acids at all and simply a low oxygen tension—something that approaches anaerobiosis although it doesn't actually have to be absence of oxygen—suffices to kill tubercle bacilli. That is, if we know what we mean by kill. It suffices to render tubercle bacilli noninfectious and noncultivatable within a period of about three or four weeks outside the body. This is in a liquid medium; of course, it also has been done on solid medium; and this matter simply of lowered oxygen tension seems admittedly to be a very central point. There are other things, obviously a great many things, that accumulate in the cavity, and organic acids are among them. These may be produced more in a necrotic cavity than they would be in viable tissue or even in inflamed tissue, or they may not. I think perhaps they are. But, if they are, this also would depend first upon the lowering of the oxygen tension. So here you have two factors, at least I think there are two factors, which might give us the kind of results which have been described here this afternoon, a low oxygen tension which in itself appears to be lethal, and, in addition to that, some of these other factors which help along lethality.

DR. HOBBY: Thank you, Sidney. I think that, in all fairness to Dr. Dubos, we should say that Sidney has brought out the point which I believe Dr. Dubos would have put first in his discussion, whereas we failed to do so. He has emphasized over and over again the importance of the degree of oxygen tension, so that it is well worth while to keep this in mind.

DR. MCDERMOTT: And the pH; that is, Sidney quite properly corrected me when I said that incredibly small amounts—it all depends upon the pH, which in a lesion is in a sense a function of the oxygen tension to a certain degree. Do you agree with that, Sidney?

DR. RAFFEL: Yes.

DR. HOBBY: Nick, if I remember rightly, you did some studies also to demonstrate whether or not any of the antimicrobial agents which were used in treating these patients could be detected in these tissues. Is there enough there to perhaps inhibit these cells?

DR. D'ESOPO: We didn't do that work, Gladys.

DR. HOBBY: Do you recall the work of Dr. Weiser which was reported at the last VA meeting? If I remember rightly, he showed that the concentration of streptomycin depends largely upon the time at which surgery is done in relation to the last dose of the drug. Nonetheless, he was able to demonstrate concentrations as high as 10 to 20 γ per ml. of his tissue suspension. I do not recall just how he suspended his tissues so that a concentration in terms of volume is perhaps not too clear; but, nonetheless, there were very definitely demonstrable quantities of streptomycin still residing in the tissues, and, of course, that in turn might have some inhibitory effect upon these cells.

MR. STEENKEN: Gladys, that would only be effective as far as culture goes, but, as soon as you put that material into animals, that small amount of drug soon dissipates

itself and the organism would then have a chance to produce disease. We have taken much of this material and washed it several times to be sure we rid it of any small amounts of residual drug. You must remember that even in patients who do not have closed lesions, who are taking drugs, there is a fair amount of drug still in the sputum and by ordinary cultural techniques, in most of the cases, we are able to grow the organisms or produce disease in animals.

DR. HOBBY: I think that's very true, Bill, but is there not a possibility that the contact of these organisms with the antibiotic, at the same time that they perhaps are in contact with some of these other growth-inhibitory factors which we have mentioned, might slow down their metabolism so that they would revive with less ease?

MR. STEENKEN: I mean, if its speculation; I have no proof.

DR. D'ESOPO: Dave, what would you say on that, as one who has studied the metabolism of cells?

DR. SMITH: Yes, that's speculative, but I don't see how we can get a hold of him. I'd like to make a general biological remark here. If you inoculate a few human tubercle bacilli into the guinea pig, the animal almost invariably dies. If you inoculate considerably larger numbers of human tubercle bacilli into a rabbit, the rabbit will survive. He would eventually kill off all of those tubercle bacilli. Dr. Burke's studies, where he followed the rabbits for some years after a very large dose inoculated intratracheally, showed that the rabbit has the capacity of eliminating the tubercle bacilli. As I recall, in many of these animals on serial section there was no evidence that there had ever been any tubercle bacilli there. Yet there were perfectly good X-ray lesions at one time. Now the man is not like the guinea pig. The man is more like the rabbit. You can kill all of a series of rabbits if you give a large enough dose of human tubercle bacilli. You can have practically all of the rabbits survive if you give a sufficiently small dose, and the bacilli are eventually eliminated in those rabbits. Now, man is, as I say, much more like the rabbit, and there is probably a basic mechanism, first, for arresting the multiplication of tubercle bacilli in man; second, rendering them noncultivatable and noninfectious, and, finally, eliminating them altogether. We probably just accelerate this natural mechanism by our antibiotic therapy; and for practical purposes, not being a real expert, I think these bacilli are dead.

MR. STEENKEN: I agree!

DR. HOBBY: Well, we have two answers anyway out of the eight of us.

I can't resist the temptation to pick up at that point. Dave has brought up the question of how many cells are being inoculated into these animals, and I know Bill Steenken speaks of them as being numerous in number. Some of the rest of us have felt that they were a little more sparse than that; but I just wonder what the situation is with regard to other microorganisms. If one were to inoculate a culture medium or even the animal host, a susceptible animal, with small numbers of cells, say in the vicinity of 1 to 10 organisms per tenth of a ml. or some such volume, how often would these grow in culture? How often would they produce disease? Sidney, would you like to comment on that in relation to some of the other bacterial species?

DR. RAFFEL: Well, I can mention a few examples. I haven't rehearsed this point. The anthrax bacillus, I think all the bacteriologists here will agree, is supposed to be the prime example of a highly infectious agent, a case in which one viable organism is supposed to produce in a susceptible host progression to death. The pneumococcus is an organism which has also been taken out of a medium by single-cell isolation methods and put into proper animals and will cause death. Now you are talking about culture. I think the bacteriologists in general feel that, for what we call ordinary bacteria, each

colony which grows on a plate, for example, represents a single bacterium, so that, although there must be some accidental occasion on which an organism falls by the way, generally speaking each individual cell produces a colony.

DR. HOBBY: Do you recall offhand, so far as the people who have done single-cell isolations are concerned, with what degree of frequency they lose the cell due to what we sometimes speak of rather loosely as a "drowning effect"? I was under the impression that the batting average was not better than 50 per cent, if that high, of cells that could be made to multiply.

DR. RAFFEL: I don't have a statistical impression on that.

MR. STEENKEN: Dr. Hobby, we don't have to resort to other bacteria, because in my early days I did a lot of single-cell work when I was trying to work with the medical students to find out how soon people would show skin hypersensitivity if they had received small numbers of organisms. So by the microdilution methods, I was able to pick a single organism, five and various numbers, and then plate them and see how many of them would grow. And, although I'd say I had picked 5 or 10 organisms which I knew were in the microdroplet, as checked by the fluorescent staining technique, we were never able to grow the actual number that we fished, because we never knew if we were picking living or dead organisms. And we had no vital stains which would tell us whether an organism was living or dead.

DR. HOBBY: So you feel that the percentage of viability of the cells or reproducibility of those cells would be fairly high, and that they would all multiply?

MR. STEENKEN: No. I don't.

DR. HOBBY: You feel they would not. That has been our impression, at least some of us, that we were working perhaps with a relatively smaller number of cells and that by the law of averages they might not always multiply in artificial media. That's just a personal impression. I don't know whether the others would agree.

DR. SMITH: Dr. Hobby, I think we are getting lost here. It is well known that bacilli in the ordinary case of tuberculosis, in the fresh case—bacilli which are present in too small numbers to be seen by smear at all—regularly produce tuberculosis in the guinea pig. Here our real problem is that the bacilli are present in large numbers by microscopic smear and, when you use an appreciable amount of that tissue, you are using an enormous amount and those have failed to grow and have failed to produce disease.

DR. HOBBY: Well, that's our point, Dave. Are they in large numbers?

DR. SMITH: Well, let's go back to Steenken, because he didn't answer the question which I want answered. As I recall, Petroff was able to kill guinea pigs with 10 human tubercle bacilli. And then we will ask him how many he thinks he inoculated and make some calculations from these smears and the amount of material he put in and failed to get growth.

DR. HOBBY: Bill, is there anything you would like to say on that?

MR. STEENKEN: Well, along with the culture work which we did on single cells, we also made small incisions in the inguinal region of the guinea pig because the micropipettes are so fragile that one cannot stick them through the skin—but we would drop the microdroplets into the incision and then suture up the incision. And with as few as one organism at times, or five organisms, we were able to produce generalized tuberculosis in animals—and eventual death. Now, when you use small numbers of tubercle bacilli to infect a guinea pig, you get a slowly progressive disease; it's not a rapidly progressive disease. The animal becomes vaccinated at the same time that the disease progresses. Therefore, you get more of a chronic type of tuberculosis and it takes a much

longer period of time before the animal dies of tuberculosis. When you examine a smear—as closely as I can calculate—there are about 300,000 microscopic fields to be examined. And in any single microscopic field, when you say they are "uncountable" or there's a Gaffky X, that means you've only taken a small amount of the specimen, a speck of the specimen, to put on the slide—so in that material you have billions of organisms. And that is one of the things that has bothered me in this whole problem. When you are dealing with the chance that these organisms may still be living out of a population of billions of organisms, we should be able to produce disease in animals or we should be able to grow these organisms. And, it's hard to believe that, in a population of organisms as great as that, a drug has so-called sterilized these organisms, or put them into the Dubos-state, such as the rooster who has been castrated and becomes a good big capon. I mean, we don't know how to do that with the tubercle bacillus. On the other hand, I just feel that, when you see populations like that which you can't grow and with which you can't produce disease even after the drug has been removed for a long period of time, I still agree with Dr. Smith that they are dead.

DR. MCDERMOTT: Dr. Hobby?

DR. HOBBY: Yes.

DR. MCDERMOTT: I think we might liven this up. I wonder if the microbiologists realize the implications of what they are saying. If things are as they say they are, then they have attained a biologic cure of tuberculosis in an individual patient. In other words these bacilli are dead, they are no longer parasites, the problem in the lung is nothing but that of a foreign body which can be handled with phagocytic cells and calcification. Moreover, in terms of the patient, it doesn't make a bit of difference whether some of them are dead as long as some of them are alive. And we have been talking thus far in "all or none" terms. I gather that it is Dr. Smith's opinion and Bill Steenken's opinion that, in these lesions which have been emulsified and injected into animals, all of the bacilli are dead. If they are not, there remains in comparable lesions which were not resected viable bacilli potentially capable of producing disease. Now in so far as the number of bacilli which are concerned to produce disease in animals, I think it would be giving a grossly wrong impression if we gave the idea that it was all quite as nice as we have heard. Everybody knows that, if you change a parasite from one type of environment to another, it does not always survive so well. We have been talking about 1 to 10 bacilli taken out of a culture medium, in a nice laboratory, by a good worker, when the wind is right, everything is perfect, and putting that in animals; and what we are talking about in lesions is taking a "beat-up" lesion with all these things in it which have been discussed, perhaps with some drug, perhaps the bacilli have drug in their enzyme systems which can't be washed off; we have a wide variety, a heterogeneous population, and we can't say whether it takes 1 or 10, it may take 100,000 in those circumstances. So, however much I would agree that the single cell observations are "the only wheel in town," I do not believe that they are strictly germane to the problem of whether all or none of the bacilli in the lesion are capable of producing disease.

DR. SMITH: Dr. Hobby, I'd like to get back in on this. My statement that these lesions with large numbers of stainable bacilli which, when cultivated in a proper medium or inoculated into animals, will not grow, those bacilli then are the ones that didn't grow. Now, I would like to emphasize again from a slightly different point of view what Dr. McDermott was saying. You have no assurance whatever that another lesion which you left in that man, which is very much smaller than the one you took out, doesn't contain living bacilli. All we are saying is that the one you took out doesn't have living bacilli. I think it is quite possible that another lesion 5 mm. away from your line of

incision has living bacilli in it and we don't know anything about that. That's not what we started out to discuss; that's another problem, a very important one.

DR. HOBBY: Thank you, Dave. I think that problem should be mentioned although I am afraid that the microbiologists are rather tied up with the question of each individual lesion and its organisms. Walsh, do you have anything to say?

DR. MCDERMOTT: Yes. I am going to say that people can swallow cultures of typhoid bacilli, people can take cultures of gonococci and put them into the urethra of normal males, and they don't get a high percentage of takes of gonorrhea; it all depends on how you do it. And the idea that these lesions which have been resected are necessarily cured is Ehrlich's dream, but one which has not been accomplished regularly in any other infection; and I certainly doubt if it has been accomplished in tuberculosis. Moreover, I think it would be very dangerous if we managed our patients on the assumption that it is true. Some of the bacilli are dead, sure.

MR. STEENKEN: I am sure that no phthisiologist would manage his patient on the assumption that the organisms are all dead. And I personally want to make it very clear that I am not trying to make a statement that all of these organisms are dead, but I am willing to make the statement that the major portion of these organisms according to present day criteria are dead. It is hard for me to go along with Dr. McDermott when he says that, out of a population of 100,000, it may take so many to produce disease. There is no question about these lesions having billions of bacilli which we can't grow and which don't produce disease. It is not a question of dealing with hundreds or thousands; you are dealing with billions.

DR. MCDERMOTT: Well, I could not agree with you more, Bill; that it is a very interesting biologic question. I simply maintain that to reason from experience in one set of circumstances to what is, in effect, a totally new area of scientific investigation, admittedly is the only way we can do it; but we are not necessarily correct in our assumptions.

DR. HOBBY: I think that just for the record and at the expense of antagonizing my good friend, Bill Steenken, I would like to say one thing, and that is: In the 6 hospitals reporting last February at the VA meeting, if I remember the figures correctly, some 67 per cent of the lesions reported were described as having Gaffky's of I to IV only, with 10 per cent being higher than IV. I believe the remainder were either negative or unreported. I think those figures are accurate. Now a Gaffky IV, as I understand it, is only about 2 or 3 bacilli on the average per field, so that it is not necessarily in the billion classification, although certainly, according to normal bacteriological standards, there should be plenty of organisms there to give growth. Bill, did you have a question or comment?

MR. STEENKEN: I want to comment on what you said. This material, you must remember, is not being concentrated as a concentrated sputum, where you can get rid of all the extraneous matters and just have the remaining bacilli; these smears are made when you pick up a small amount and put it between two microslides and you drag it out. In that material, as I said before, there are approximately 300,000 microscopic fields. If you admit that a Gaffky IV represents two bacilli per field, even on that slide, if you multiply 300,000 by 2, you would have 600,000 bacilli. So you multiply it by the millions, the small amount of material that is on that slide; in that caseous lesion you are going to get into the billions.

DR. HOBBY: Bill, how many viable bacilli do you normally—take, for example, the H37Rv strain—use to infect a guinea pig?

MR. STEENKEN: Well, once upon a time, before we had a good antibiotic that could

practically cure an animal of the disease, we were very careful to use a selected dose because, if we used too many organisms, we got death. If we were trying out, say BCG or any other vaccination, the question was always, if we gave too large an infecting dose we would kill our animal too soon, so we used small numbers, but today with antibiotic agents you just pour bugs in there and nothing happens.

DR. HOBBY: You haven't answered it.

MR. STEENKEN: That was the answer.

DR. HOBBY: How many viable cells? I think it's in the vicinity of 40 million.

DR. SMITH: No. No.

DR. HOBBY: Yes, by the technique he taught us.

MR. STEENKEN: Well, with some of the techniques, if you want to use the plate count methods, knowing the fallacies in such laboratory techniques. I don't think we have any method today that can detect the actual number of viable cells in any suspension.

DR. FELDMAN: I'd like to ask some member or more of the panel what is the state of the hypersensitivity to tuberculin in these individuals in whom you are unable to demonstrate viability or virulence from these so-called healed lesions. There is some evidence, for instance, that in the Ghon complex, in which you get practical uniformity of tuberculin, or positive Mantoux reactions, as these individuals get older (providing first that they have not been exposed or subjected to any exogenous reinfection) a percentage of these individuals lose their tuberculin hypersensitivity. I think this would be an interesting point on which to comment.

DR. MCDERMOTT: I think the answer is what Dave Smith said, namely, that the lesion next door might have viable bacilli in it which would produce hypersensitivity. Incidently, of course, we're guilty in the heat of the moment of a panel of using words incorrectly. The *question* is whether these bacilli are viable or not; what they *are* is nonculturable.

DR. HOBBY: Yes, Nick.

DR. D'ESOPO: We have several clinical observations which might help clear up these microbiological speculations. We have now followed patients for three years who have reached the "target point" but for one reason or another have not been resected, mostly for reasons of age and associated diseases. As far as we can judge, they had reached the point at which, if they had been resected, we would have had difficulty in growing tubercle bacilli. Now the follow-up of these patients is exceedingly good; at least these patients act as if the bacilli are dead. In addition to that, there are a few patients who have been resected and the tubercle bacilli have not been grown from the lesions, but they have had a positive culture directly after surgery—instances in which the surgeon felt he had taken out all of the palpable lesions. These lesions have not grown out and yet tubercle bacilli have been found. We have taken this to mean that under laboratory conditions there may be a few bacilli which cannot be cultivated. What did you say, Walsh?

DR. MCDERMOTT: Well, I just want to make a point, Nick, that in the days before antimicrobial therapy, we certainly had people who left sanatoriums and went back to very active lives and lived those lives for five or ten years or fifteen years and then subsequently developed overt pulmonary tuberculosis. We did not regard them as fresh infections; we regarded them as relapses of the tuberculosis which they had when they left that sanatorium. So I object to your saying the patients behaved as if the bacilli are dead. I just don't think that is justified.

DR. D'ESOPO: Well, I'll say it again. Thus far, after three years of follow-up, the

patients have behaved as if the bacilli are dead. In addition to that, I think your statement about late relapses, after 15 years, is not right. The percentage of relapses after the fifth year is very low indeed.

MR. STEENKEN: Gladys.

DR. HOBBY: Yes.

MR. STEENKEN: I think that the answer to Walsh's question is that we can't hit a patient over the head at any one period of time and take all of the lesions and all of the lung material. But in animals we have been able to create these types of lesions; we have been able to produce filled-in cavities; we produce under controlled conditions the identical thing which is seen in human beings. We can autopsy the animals and get the same material and we are working in the same host where these organisms produced disease. So to me, I think that that serves as a basis for those who want to challenge this phenomenon, who believe that these organisms can be revived; I don't know, if we try everything you can think of at present, are we still going to say that, if we wait long enough, we will gradually be able to grow these organisms. I mean, I think it's wishful thinking.

DR. HOBBY: Well, Bill, you have led right up to another group of questions and I think we might go on. Obviously, we don't all agree with each other at this point, but that's all to the good too. Sidney, you have discussed this point with us before and might like to again. Is there any general evidence that infectious agents may exist in dormant form for periods of time and then regain their viability? What about some of the other bacterial species?

DR. RAFFEL: Well, at the risk of saying more than I am asked to say—I think most of the audience are probably not microbiologists—it might be well to state this simple fact. There is reason for suspecting organisms of being capable of revival because under certain circumstances certain microbes are capable of revival when they seem to be dead. In the past two or three or four years, it has been found, for example, that ultra violet treatment of certain bacteria, which apparently kills them, actually doesn't kill them, because a large percentage of them can be revived on exposure later to visible light. And it has been known that certain organisms which apparently are killed by bichloride of mercury, which we all certainly think of as a good lethal agent, can be revived to a degree by hydrogen sulfide, for example. I want to put that on the record because I think that otherwise the discussion might be very academic—it's pretty academic anyway. Then as to the question you ask, the basis for suspicion of dormancy. I just jotted that down here too. There are certain agents in the body which appear, on pretty good evidence, to be able to simply exist in the body for unlimited periods of time, apparently without doing anything except existing. One of them is a virus, herpes simplex virus (fever blister virus), which, and I think that there is general agreement amongst virologists on this, causes a life-long infection, and all of you are acquainted with the fact that,—especially in certain individuals,—you never see any signs of that infection. But when you look for the virus in just about any member of the population, you find it in epithelial cells. It's not a bacterium, but it is a living infectious agent. I think perhaps the spirochete of syphilis could be looked at in the same way. It's a little bit vague here, and a little bit less tenable, but there is good reason to believe that these organisms do persist, as all of you know, for many years, and apparently without doing a dog-gone thing until they later become reactivated.

DR. MCDERMOTT: How about Brill's disease?

DR. RAFFEL: Well, Brill's disease is another example I think. There is fairly good evidence that Brill's disease, as Dr. McDermott suggests, is a residuum of some long-ago, rickettsial infection, without any signs of infectiousness for many years. And then,

if I can take a moment more, I would like to say that yellow fever might be a very good example of this. There is a fascinating story of how some years ago people in non-yellow fever sections of the world—New York specifically—where individuals who had been exposed to the disease as long as seventy-five years before in one case and fifty years in other cases still retained antibodies against yellow fever virus. Now, of course, we can say that they had infections seventy-five years ago or fifty years ago and they continued to make antibodies all this time against nothing—just the memory of the infection. It doesn't seem very likely; it would seem much more likely that the yellow fever virus persisted for many years, for the lifetime of the individual, in a viable form. Not a very good answer to the question but—

DR. HOBBY: Do you believe that there is any similar evidence with regard to the tubercle bacillus?

DR. RAFFEL: I hate to say this because I am very unsure of my ground and many in the audience will disagree with it, but I think the epidemiological situation in tuberculosis, the fact that reinfections occur in individuals, I mean that most clinical tuberculosis occurs as a reinfection type and occurs usually in an area of the lung where the influence of gravity doesn't enter in, would surely indicate that most of these infections must be endogenous reinfections, and the occurrence of an endogenous reinfection presupposes that there were bacteria around in the body to start it. I am sure that we can get a pretty good argument on this story here.

DR. HOBBY: Walsh, you had some thoughts on instances in which apparently dormant cells have been revived. Would you like to comment further on this?

DR. MCDERMOTT: Well, I think Sidney has covered it very well. There are innumerable examples in infectious diseases of carrier states and long periods of dormancy with the eventual eruption into full-scale clinical disease. Indeed, the cortisone situation in humans and in animals is a very good example in point. For example, in one situation one cannot cultivate a particular microorganism from a rat; one can give the rat cortisone and the organisms emerge to produce a fatal disease. The general biologic principle that latent infections can persist for a period of years or decades is so well established that I don't think really any further examples are necessary. Now, in so far as the tubercle bacillus is concerned, as Sid said, it's a debatable point and one can look at it either way. I would like to make one comment though, from the field of other infections, on this point and it has to do with my opening comment. It is a common clinical experience, and I think Dave can back me up on this, that one will get a patient in the hospital with an acute infection,—let us say, the characteristic picture of acute pneumococcal pneumonia and empyema,—but the patient will have received a day or two of penicillin but will still be sick. One can aspirate that pleural fluid and at times one can see microorganisms on stained smear but cannot culture them. If one washes one's hands of the situation at that point, the patient behaves like any other patient with an acute empyema.

DR. HOBBY: Dave, is there anything you would like to add?

DR. SMITH: Yes. I made the statement earlier that I thought these lesions contained large numbers of tubercle bacilli which were not cultivatable and could not kill the guinea pigs and which were dead. I want to make it very plain that I do not draw the conclusion from that that all of the bacilli in that man are dead. Certainly the old nursery rhyme about Rover, when he died he died all over, doesn't apply to tuberculosis.

DR. MCDERMOTT: My point is, I am not sure it applies to one bacillus, Dave.

DR. SMITH: Well, I'm inclined to think those which won't grow in the guinea pig, for practical purposes—there I am with Dr. D'Esopo—for practical purposes, they have reached a biological balance which isn't going to hurt the man anymore. I am not at

all sure that another lesion in a lymph node or another part of the lung wouldn't grow readily and kill an animal and cause relapse and kill that man. So from the public point of view, from the general doctors' point of view, we must avoid a conclusion that the man has been sterilized as far as his tuberculosis is concerned just because the particular lesion we took out has been sterilized. I'm willing to grant that, but I am not granting that you have gotten all the viable tubercle bacilli out.

DR. MCDERMOTT: And in the use of words, I couldn't agree with you more that the lesion has been sterilized, because by sterile I understand you to mean that the bugs won't grow. My point is they may still be alive and may be capable of growth at some future date.

DR. HOBBY: There's been some very interesting work recently which I think we ought at least to mention before our time this afternoon comes to an end, work related to the ability to reverse inhibition of growth, and I think we might turn to that subject at this point. Walsh is there anything you would like to say with regard to the substances that have been used to reverse inhibition of growth?

DR. MCDERMOTT: Well, no; actually you know more about that than I do, Glad. Penicillinase; the recent work that would indicate that ketone bodies would reverse the inhibition of organic acids at low pH on *Staphylococci* and tubercle bacilli; and then the things which Sidney mentioned—the H_2S with mercuric chloride; and an ultraviolet "killed" culture can be revived if you just put it on any old electric light bulb.

DR. HOBBY: Dave, are you familiar with Dubos' work with regard to the keto acids? I believe that he has found that organisms, if I am quoting him correctly—and I hope I am—he has found that organisms maintained at a pH around 5.5 or 5.6, which very quickly under suitable conditions prevents their subsequent multiplication, can be reversed by the addition of certain of the keto acids. There is also some work which has been done by others which I believe indicates that sodium pyruvate can reverse inhibition of growth under suitable conditions. Donovick has come out with some work recently in relation to other of the antimicrobial agents. Is there any of that on which you would like to comment, Sidney?

DR. RAFFEL: No. I wouldn't.

DR. HOBBY: This is all a new field but it suggests the possibility that perhaps we don't know what is the optimum set of conditions under which to cultivate these organisms which have been in the host for varying periods of time. There is also the question of mutation which perhaps warrants some comment. Walsh, you are familiar with the work that has been done by Dr. Wesley Spink's group with the *Staphylococcus* recently. Are there any parallelisms here or possible parallelisms?

DR. MCDERMOTT: Well, I think it would be just another speculative mechanism. They have found a microcolony of *Staphylococci*, penicillin-resistant *Staphylococci*, but they were able to cultivate the *Staphylococci*, which, as Bill Steenken pointed out, is not the case here. You simply cannot cultivate the tubercle bacilli in these situations.

DR. HOBBY: Now, before we bring this to a close, so to speak, I would like to ask one more question. Dave, you commented about the fact that the Warburg technique perhaps is not too applicable here. What is your thought with regard to the tetrazolium test or other techniques of that sort?

DR. SMITH: Well, there is work being done on that. I've not done any myself. If properly controlled, the use of tetrazolium might indicate metabolism in the organism in the absence of growth. I think there was a report that they did not show these things. Wasn't there—at the meeting in Atlanta?

DR. HOBBY: I'm not too familiar with those results, except that any technique that

might demonstrate that the cells still can respire of course would be of tremendous interest in this connection.

Well, I think Walsh might summarize for us some of the thoughts that have been expressed at this time; and there is one last question that we would like to throw out for discussion, and perhaps you would care to comment on it. The question has been posed: If it is true that tubercle bacilli in the so-called healed lesion are in a state of quiescence, doesn't the failure to grow or to exhibit virulence in the highly susceptible host impugn the reliability of cultures and guinea pigs in certifying the diagnosis of cases of clinical tuberculosis?

DR. MCDERMOTT: In an attempt to summarize this discussion, I think one can say that the facts are clear and simple. The organisms, which bear every characteristic of being tubercle bacilli on direct observation, are present in the lesions which are resected, and by a very careful series of methods it has not been possible to culture the organisms on standard culture mediums. And make no mistake about this: The people who have been attempting to do this know their business, and they have been unable to culture the tubercle bacilli. So I would say that, as some of the members of the panel expressed themselves, at least half the panel, and perhaps a majority of the panel, believe that in those situations the lesion has been sterilized. I would agree that they have been sterilized, that's just by definition; but they believe that the organisms are dead. Others in the panel, or at least I, are by no means convinced that the organisms are indeed dead or that any large numbers of them are dead, and believe that a proportion at least are capable of revival. The arguments in favor of the fact that they are dead all consist of the fact that it has not yet been possible to culture them; those are the only observed facts. The arguments that they may be capable of revivication are based by analogy on general biologic experience. Dave Smith has made the point, which is a very important point, that microbial populations are heterogeneous affairs and that there is too great a tendency to regard every single member of a microbial population as being in exactly the same physiologic state at any point in time; whereas it is perfectly possible that some of them can be very lively, some of them tired, some of them dead, and some of them completely indifferent to what is going on. In any event, at least half of the panel, perhaps all but myself, believe that these cultural methods are adequate and that the organisms are dead. I dissent from that point of view.

Insofar as the question you have asked, Gladys. I think that the only proof in this type of situation is positive proof. I think that, if you can show the bugs and show that they produce disease, you have got something. Negative results, however impressive, do not really answer the question. In any series of comparative studies in which cultures and inoculations of guinea pigs have been made, there are always a few pigs which were positive when the cultures were sterile and there are always a few cultures which are positive when the guinea pig does not show any disease. So there isn't an absolutely precise one-to-one correlation there.

Insofar as saying what about our guinea pig and culture methods, I think all we can say is they are the best we have; we would like to have better.

And finally, on the contra side of the argument, is the point that it is conceivable that the cultural methods which have grown up for the culture of sputum and other body exudates might not be the best cultural methods for the isolation of tubercle bacilli which have been exposed not only to the noxious agents of the drug but also to the presumed noxious agents of the lesion. I think the majority of the panel are in agreement that the bugs are dead.

DR. HOBBY: Thank you, Walsh. In other words, we find ourselves much in the same position that the leprologists have been in for years. Simply that their organisms can be stained, they are present in abundance, certainly in murine leprosy at least there can be no argument about the large numbers of cells, and yet they have not been able to cultivate them. And that leaves a certain amount of doubt in the minds of many of us as to what the situation may be insofar as the tubercle bacillus is concerned.

I am sorry that there isn't time to hear final comments from some of the rest of you, but, with that, I think we might thank the panel members for their help today and for giving us their thoughts and thank the audience for listening to us.

Report of the Ninth Pembine Therapy Conference Michigan, Wisconsin, and Minnesota

The ninth Pembine Therapy Conference for pulmonary diseases was held at the Four Seasons Club, Pembine, Wisconsin, September 5, 6, and 7, 1952.

Invitations to the conference were issued in the same manner as in former years.

In general, the pattern of previous conferences was followed. The tuberculous cases for the area sanatoriums were presented by the Nopeming Sanatorium, Nopeming, Minnesota; Maybury Sanatorium, Northville, Michigan; and Muirdale Sanatorium, Milwaukee, Wisconsin. For the 1952 conference, the cases of twenty-five consecutively treated tuberculous patients who received collapse therapy or surgical procedures after January 1, 1952, were presented.

Also, following the practice of former conferences, a similar series of cases from a selected sanatorium from outside the area was presented. These cases were presented by the Barlow Sanatorium, Los Angeles, California.

For 1950, 1951, and again for 1952, the scope of the conference was extended to include nontuberculous chest cases in separate presentations. These nontuberculous cases, representing twenty consecutive major thoracic surgical cases, excluding tuberculosis, cardiac cases, and bronchogenic carcinoma, were presented for Michigan by Dr. William Tuttle from his practice; for Minnesota, by Dr. Robert Schmidt from the practice of Dr. Thomas J. Kinsella; and for Wisconsin, by Dr. Paul Hausmann from his practice.

Representative roentgenograms and data presented by the presentor and submitted to the conferees in table form were reviewed and discussed.

Following presentation and discussion of cases, each presentor summarized his material.

The following guests from outside the conference area were in attendance and contributed greatly to the conference with their discussions and constructive criticisms:

John B. Barnwell, Washington, D. C.
Daniel E. Jenkins, Houston, Texas
John C. Jones, Los Angeles, California
Hiram T. Langston, Riverside, Illinois
William H. Oatway, Altadena, California
Edward L. Ross, Winnipeg, Manitoba, Canada
William Steenken, Jr., Trudeau, New York
John W. Strieder, Brookline, Massachusetts
Henry C. Sweany, Tampa, Florida

The first day of the conference was devoted to presentation and review of the tuberculous cases within the conference area as above noted.

The following therapy trends were noted in the presentation of these cases:

(*a*) Only one pneumothorax had been initiated, and this case was in the Michigan series. It is to be noted that this patient had refused resection.

(*b*) There were four phreniclases. All of these were in the Michigan series.

(*c*) There were two initiations of pneumoperitoneums, and both of these were in the Michigan series.

(*d*) There were fourteen thoracoplasties, four in the Michigan series, seven in the Wisconsin series, and three in the Minnesota series.

(*e*) There was one extrapleural pneumonolysis with paraffin, which was in the Wisconsin series.

(*f*) There were three thoracotomies for decortication. Two are listed in the Minnesota series, and one in the Michigan series.

(*g*) Resections. Of the 75 tuberculosis cases presented by the tri-state area, pulmonary resections had been performed in 52. In some instances, the resections were multiple and bilateral.

Michigan—14 patients	
Wedge resections	3
Segmental resections	5
Segmental and wedge resection	1
Lobectomy	4
Pneumonectomy	1
Wisconsin—21 patients	
Wedge resections	15
Segmental or lobar plus wedge resection	6
Minnesota—17 patients	
Segmental resections	13
Lobectomy	3
Pneumonectomy	1

It is to be noted that, in the report covering the 1951 conference, there were a total of eleven resections among the eighty cases presented.

The following observations were made covering presentations of tuberculous cases from the tri-state area:

It was generally agreed that antimicrobial therapy for at least four months is indicated prior to resection. The period of postoperative antimicrobial therapy varied considerably depending on preoperative therapy and extent of disease.

There was no defense of either pneumothorax or phrenic nerve interruption alone by any of the conferees or any of the presentors. There was some thought that phrenic nerve interruption and pneumoperitoneum was still of value. There was evidence of marked decrease of patients coming to thoracoplasty, and it was the thought of the presentors that many of the present cases with thoracoplasty would now be submitted for resection.

There was general agreement that for the present, at least, residual caseous foci should be resected with long-term antimicrobial therapy.

It was emphasized that the ultimate relapse rate in these cases would be the criteria rather than just tissue bacteriology of the resected specimen.

There was consensus that streptomycin (1 gm.) two or three times weekly and PAS (12+ gm.) daily in general represents the best known antimicrobial program (at the time of the conference).

It was emphasized that the findings at exploration and in the resected specimen were often greater than the involvement anticipated from the appearance on the chest film.

In the Muirdale series it was reported that patients who had undergone pulmonary resection procedures were being discharged three months after operation. There was considerable discussion and opposition to this program except in those areas where such handling is dictated by the scarcity of available beds. The presentors pointed out, however, that the program is being carried out as a pilot study.

Postoperative persistent peripheral lung leaks in the Muirdale series were managed by either thoracoplasty or secondary repair. There was, in general, a conviction by the presentors, particularly in the Wisconsin group, that the imme-

diate preoperative roentgenographic appearance is by no means the most deciding factor for resection, but rather the original and pre-streptomycin disease.

The problem of treatment of the elderly patient was discussed. It was thought by many that these patients tolerate resection quite satisfactorily.

There was considerable discussion of the problem of carbon dioxide retention during intrathoracic surgery. It was stressed that oxygen alone does not materially help such patients, but that deep breathing and positive pressure should be used. Tracheotomy may be indicated for removal of secretions and the reduction of the relative dead air space in patients who are having respiratory difficulty.

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Dr. William Oatway, assisted by Dr. John C. Jones, from the Barlow Sanatorium, Los Angeles, California, presented twenty consecutive surgical cases in accordance with the above pattern.

The Barlow group prefers lobectomy, if possible, to multiple wedge resections.

There was criticism of the cases presented for maintaining pneumothoraces, particularly in the presence of adhesions.

It was noted that chemotherapy was used for shorter periods of time than in the Pembine area. It was brought out in the discussion that in certain local situations such determinations are beyond the control of the staff.

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Dr. William Tuttle presented twenty major thoracic surgery cases from his private practice. These consecutive cases were after December 1, 1951.

The complications of esophageal resections were discussed, with particular reference to the occurrence of peptic ulceration.

There were those who advocated the use of pyloroplasty to hasten stomach emptying.

The resection of any round lesion was strongly advocated by the presentor. Cases of esophageal diverticulum, middle lobe atelectasis, chondroma, empyema, pulmonary cysts, fibrosarcoma, substernal thyroid, diaphragmatic hernia, and sarcoid were presented.

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Dr. Paul Hausmann from Milwaukee, Wisconsin, presented twenty consecutive surgical cases from his private practice.

There was considerable discussion of three cases of sternum excavatum corrected by surgery. There was general agreement that surgery should be carried out at an early age. Best results are obtained before the age of eighteen months.

The value of thorough evaluation of the bronchial tree preoperatively in bronchiectasis was emphasized. In one case there was residual bronchiectasis left in the superior lingular segment which was not demonstrated in preoperative bronchograms.

Cases of spontaneous pneumothorax, thymic tumor (causing a Horner syndrome), a 25-year-old empyema, sternum excavatum, lung abcess, carcinoma, bronchial cyst, and bronchiectasis were presented.

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Dr. Robert Schmidt presented twenty surgical cases for Minnesota from Dr. Kinsella's private practice.

The near necessity for a preoperative period of medical management for bronchiectasis was emphasized.

With bilateral bronchiectasis, the question was raised whether one side should be operated upon even if it is thought that an eventual operation on the other side would be impossible. The presentor believed that the unilateral procedure should be done in such circumstances, with the hope of improvement and relief of symptoms.

Cases of bronchiectasis, granuloma, mesothelioma of pleura, dermoid (left mediastinum), bronchial adenoma, hamartoma, funnel chest, neurofibroma, pharyngeal esophageal diverticulum, obstructed and infected emphysematous bleb, dumbbell-type lipoma (right chest wall), and multilocular cyst (pericardiophrenic angle) were presented.

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GUEST PRESENTATIONS

The Results of Recent Work with Isoniazid

William Steenken, Jr., Trudeau, New York

Isoniazid is a potent addition to the antimicrobial therapy armamentarium.

Less clearing and less rapid clearing was noted in rabbits following the use of isoniazid than in the streptomycin series.

The difficulty or inability to grow, on culture or in animals, the acid-fast bacilli found in resected caseous foci following long-term antimicrobial therapy with either isoniazid or streptomycin and PAS was emphasized. It was pointed out by the presentor, however, that tubercle bacilli have a natural dying rate in the body, and dead bacilli can be found in persons who have never had antimicrobial therapy.

Slides shown by the presentor illustrated the recurrence and extension of infiltrations in rabbits following the cessation of antimicrobial therapy.

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Discussion of Antimicrobial Therapy

Ross McLean and William Tucker of the Veterans Administration

A comparison of randomized cases treated with isoniazid or streptomycin and PAS was presented:

During the early months of treatment there was little difference in the two groups measured by the general condition, roentgenographic and bacteriologic studies, although streptomycin and PAS appeared to have a slight advantage.

Thus far, streptomycin and isoniazid (300 mg. daily) have appeared to be less effective than streptomycin and PAS.

Streptomycin and PAS generally do not appear to be as effective as in the reported results in 1948, probably due to differing characteristics of the patients treated in the two time periods.

Seven Veterans Administration hospitals reported that apparently 10 per cent of the patients on isoniazid alone experienced an extension of their lesions. (This study is incomplete.)

Of 108 patients on isoniazid alone after four months, tubercle bacilli from 51 per cent were resistant to 5.0 γ per ml.

After four months of isoniazid alone, there were 15 resected lesions from 108 patients, 14 of which yielded cultures positive for tubercle bacilli.

Bacteriologic studies were as follows:

Sputum smear negative for tubercle bacilli:

Streptomycin-PAS	40 per cent
Isoniazid	29 per cent

Marked to moderate roentgenographic improvement in seven to ten weeks:

Streptomycin-PAS	15 per cent
Isoniazid	7 per cent

In any given period of treatment up to three months, the percentage of roentgenographic improvement, general improvement, and bacteriologic improvement was greater in the streptomycin group, but not significantly so.

Toxic reactions to isoniazid: No significant evidence of isoniazid toxicity to the hematopoietic system, liver, or kidneys was noted. Albuminuria has been noted in a few cases. Consistently, there have been euphoric mood changes. No severe asthmatic reactions have been noted.

Discussion of the presentations by Drs. Tucker and McLean brought out the experiences of others with the use of isoniazid. Of particular significance was the number of cases of mental confusion and urticaria. In discussing this presentation, Dr. Sweany of Florida emphasized the hitherto unreported increase of blood urea nitrogen in 50 per cent of the cases observed by himself and his associates.

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Pulmonary Ventilation

William Stead of Minneapolis

During surgery, arterial blood pH and carbon dioxide studies were done within one-half hour of the time surgical patients were returned to the ward.

The arterial blood pH should be expected to return to normal in about three hours. An example of the fluctuations is noted in the following case: The patient had an arterial blood pH of 7.405 on the day before surgery; there was a maximum drop during surgery to 7.155. Arterial blood carbon dioxide preoperatively was 40 and the highest during surgery was 82. Alveolar ventilation was approximately half of the preoperative value within one-half hour after entering the pleural space.

The carbon dioxide level rises proportionately if there is a concomitant drop in alveolar ventilation.

If from the beginning the alveolar ventilation is kept at a normal level, the carbon dioxide level will be properly maintained in most patients.

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Tissue Cultures

William Steenken, Jr., Trudeau Laboratory, Trudeau, N. Y.

Retention of staining properties of organisms in resected lesions does not necessarily mean that the organisms are viable. The findings in a series of 63 resected lesions from 20 patients who had been on streptomycin and PAS for eight months were reported. One and six-tenths per cent of the lesions resected were positive for *M. tuberculosis* by culture and guinea pig examination.

Cardiovascular Surgery

John C. Jones, Los Angeles, California

The presentation dealt primarily with preoperative and postoperative management. The value of adequate preoperative digitalization and avoidance of over-digitalization was emphasized.

Light anesthesia and sparing use of drugs were stressed.

The value of continuous use of electrocardiography during surgery was discussed.

Full cooperation between the cardiologist and surgeon and anesthesiologist was emphasized.

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SUMMARY

Henry C. Sweany, Tampa, Florida

In his introductory remarks, Dr. Sweany compared the current therapy trends with control programs of thirty-five years ago and reviewed the progress during his years of activity in the field of tuberculosis control.

In referring to the cases presented, Dr. Sweany stated that it is difficult to make comparisons between those areas where adequate case-finding programs and treatment facilities are available and other areas where major surgical treatment is still a last-resort procedure.

Dr. Sweany urged a one- to two-year follow-up study of the cases presented.

The discussor commented on the passing of the pneumothorax era and noted that thoracoplasty was also on the way out, but commented on the lives saved by these procedures in earlier decades.

The need for a better understanding of the many variations in the pathology of tuberculosis was emphasized.

In discussing the cases presented from outside the conference, Dr. Sweany called attention to the conservatism shown in the treatment of the cases presented. He pointed out that there was a larger number of pneumothoraces than is found in most parts of the country and that there was not evidence of a full appreciation of the value of continuing chemotherapy for longer periods of time. Although much of this variability, he thought, may be ascribed to the type of material they were obliged to work with.

From the available information, it was thought that isoniazid could not be given the same important position in the therapy program as streptomycin and PAS.

In closing his remarks, Dr. Sweany emphasized the variability of the behavior of tuberculosis and the importance of dosage, virulence, and resistance, and pointed out that even now we are continuing to see hopeless cases of tuberculosis.

George C. Owen, Wisconsin
Ejvind Fenger, Minnesota
C. J. Stringer, Michigan

NOTES

ANNUAL MEETING OF THE AMERICAN TRUDEAU SOCIETY

The American Trudeau Society, medical section of the National Tuberculosis Association, invites the submission of abstracts of scientific papers for presentation at its next annual meeting in Atlantic City, New Jersey, May 17 to 21, 1954.

Full information can be obtained by writing to the Chairman of the Medical Sessions Committee, American Trudeau Society, 1790 Broadway, New York 19, N. Y.

MICROFILMS OF THE REVIEW AVAILABLE

The National Tuberculosis Association entered into an agreement with the University Microfilms Company to microfilm the AMERICAN REVIEW OF TUBERCULOSIS for its sales and distribution to subscribers of the REVIEW.

A ten per cent royalty on total sales was returned to the NTA.

At the present time, volumes 63 and 64, 1951, of the REVIEW are available on microfilm at a charge of $5.20. Volumes 65 and 66 for 1952 are available at the price of $5.00.

Orders should be sent directly to University Microfilms, 313 N. First Street, Ann Arbor, Michigan.

FOURTH ARGENTINE CONFERENCE ON TUBERCULOSIS

The Fourth Argentine Conference on Tuberculosis will be held November 11, 12, and 13 in the city of Mar del Plata. Dr. Oscar P. Aguilar, President, and Dr. Jose B. Gomez, Secretary General, announce the following four principal subjects:

Tuberculosis discovered by the examination of household contacts

Tuberculous meningitis

Treatment of large tuberculosis pulmonary cavities

Antibiotics and chemotherapy in pulmonary tuberculosis

Further information can be obtained by writing to Dr. Gomez at Larrea 92, Buenos Aires, Argentina.

ANNUAL MEETING OF THE MISSISSIPPI VALLEY TRUDEAU SOCIETY—1953

The 1953 annual meeting of the Mississippi Valley Trudeau Society will be held in conjunction with the fortieth annual meeting of the Mississippi Valley Conference on Tuberculosis at the Nicollet Hotel, Minneapolis, Minnesota, October 15–17, 1953, according to Dr. J. A. Stocker, Secretary-Treasurer of the Society.

Two clinical sessions have been arranged. One will be Thursday afternoon, October 15, and the other Friday morning, October 16. Friday afternoon the Trudeau Society will meet with the Mississippi Valley Conference, when Dr. D. G. Madigan, chest consultant, Farnborough Hospital, Kent, England, will deliver a paper on "Old Tuberculin and Bacillary Emulsions under Chemotherapeutics Cover in Treatment of Tuberculosis" and Dr. J. Arthur Myers of Minneapolis will discuss "Accomplishments and Future Needs to Eradicate Tuberculosis."

At the first scientific session the following subjects will be discussed: "The Nodular Lesion," "Management of Meningeal and Miliary Tuberculosis," "Genitourinary Tuberculosis," "Report of Isoniazid Study," and "Tuberculosis Control in Industry."

Subjects at the second scientific session will be: "Bacterial Resistance to Modern Chemotherapeutic Agents," "Pulmonary Circulation in Mitral Stenosis and Alterations

Following Valvulotomy," "Esophageal Surgery," "Surgery of the Mitral Valve," and "Respiratory Acidosis."

Dr. William B. Tucker of the Veterans Administration Hospital, Minneapolis, will present a number of cases for review at the closing session Saturday morning, October 17.

MEDICAL RESEARCH FELLOWSHIPS

The Division of Medical Sciences of the National Research Council is accepting applications for postdoctoral research fellowships for 1954–1955. These awards are designed to offer research experience for promising individuals who look forward to investigative careers, and not to provide practical experience in the clinical field. Ordinarily fellowships are not granted to persons more than thirty-five years of age. The following programs are announced:

Fellowships in Cancer Research are awarded by the American Cancer Society on recommendation of the Committee on Growth of the Division of Medical Sciences. Awards are available for study in all branches of the biological, chemical, and physical sciences and of clinical investigation applicable to the study of growth, typical or malignant. Citizens of the United States are eligible.

British-American Exchange Fellowships in Cancer Research also are awarded by the American Cancer Society upon recommendation by the Committee on Growth. They are offered to citizens of the United States for advanced study in Great Britain in specialized fields pertaining to the problem of cancer. Similar fellowships are awarded by the British Empire Cancer Campaign to young British scientists for research in the United States.

Fellowships in the Medical Sciences, supported by The Rockefeller Foundation and by The Lilly Research Laboratories, are administered by the Medical Fellowship Board of the Division. Fellows are expected to devote themselves to research in the basic medical sciences. The Rockefeller Fellowships are open to citizens of the United States and Canada; the Lilly Fellowships only to citizens of the United States.

Fellowships in Tuberculosis are also administered by the Medical Fellowship Board under a grant from the National Tuberculosis Association. These awards are designed to promote the development of investigators in fields related to tuberculosis. They are open to citizens of the United States who are graduates of American schools.

Fellowships in Radiological Research are administered by the Division's Committee on Radiology for the James Picker Foundation. The Foundation has expressed particular interest in the support of candidates who propose to carry on research oriented toward the diagnostic aspects of radiology. Appointments are not limited to citizens of the United States.

Applications for 1954–1955 under any of these programs must be postmarked on or before December 10, 1953. Fellowships are awarded in the late winter or early spring. Complete details and application blanks may be obtained from the Fellowship Office, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.

THE AMERICAN REVIEW OF TUBERCULOSIS

ABSTRACTS

Edited by LAWRENCE B. HOBSON

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CLINICAL STUDIES, TUBERCULOSIS

Lung Resection for Primary Tuberculosis.—Since most primary tuberculous infections heal spontaneously, even when accompanied by minute pulmonary infections, the indications for operative removal of such foci are limited. In many cases, however, small numbers of acid-fast bacilli can be recovered from the sputum at intervals despite apparent clinical cure. Serious results may follow if frequent sputum examinations are not made in all cases of recent primary infection. In 5 patients, aged seventeen to twenty-four years, primary tuberculosis apparently responded well to medical therapy but the sputum became positive for tubercle bacilli again after a few months to five years. Lobectomy, with removal of caseated lymph nodes, bronchial or parenchymal disease, was followed by sputum consistently negative for tubercle bacilli in 3 of the 5 cases. It rarely seems necessary to operate during the initial period of a primary infection which is carefully watched and treated but, if tubercle bacilli persist in the sputum, early radical treatment is indicated. It should not be delayed until conditions become serious.—*La résection pulmonaire dans la tuberculose primaire, C. Dijkstra, Acta tuberc. belg., December, 1952, 42: 468.*—(*A. T. Laird*)

Bronchial Tuberculosis.—True primary bronchial tuberculosis with a primary focus in the bronchial mucosa is extremely rare and of theoretic importance only. In so-called "primary" bronchial tuberculosis, the erosion of a caseous lymph node into a bronchus is the essential feature. Important clinical findings include (*1*) sudden onset of severe cough, (*2*) expectoration of caseous or calcified material, (*3*) the presence of a wheeze, (*4*) demonstrable tubercle bacilli without a detectable parenchymal cavity, and (*5*) the presence of wedge-shaped opacities on the chest roentgenogram. Bronchoscopy is the most valuable diagnostic procedure, but even this may fail in many cases of perforated bronchial lymph nodes. Hence, all patients with findings of this type were subjected to closer examination with bronchoscopy, bronchial tomography, and sometimes bronchography. At Glittre Sanatorium, Norway, during 1,262 bronchoscopies in 1,003 adult patients, 20 (2 per cent) were found to have definite lymph node perforation into the tracheobronchial tree. The majority of these patients had large, crater-like perforations from which pus and caseous material could be expressed. Other less obvious evidence of perforation occurred as mucosal holes in 81 cases. These fistulous ducts in the mucosa sometimes were so tiny that they would easily have been overlooked were not a telescope used. The surrounding mucosa was often slightly injected but sometimes there was no mucosal reaction at all. Many of these holes may have represented anomalies; others may have been diseased ducts of submucosal mucous glands; but some undoubtedly represented lymph node perforations. The site of predilection for these mucosal holes was the lower part of the upper lobe bronchus. Streptomycin and *p*-aminosalicylic acid tended to have a favorable effect on the perforations and granulations, despite some end results of bronchostenosis, often with an extensive cicatrix. When no gross aspiration had taken place, the prognosis of small perforations seemed to be good. The prognosis was more serious in large perforations. Apart from spread by aspiration, such lesions might allow prolonged excretion of bacilli, secondary

bronchostenosis, and atelectasis.—*"Primary" bronchial tuberculosis in adult sanatorium patients, P. Vakwik, Acta tuberc. Scandinav., 1952, 27: 255.—(K. T. Bird)*

Cortisone and Tuberculin Sensitivity.—The effect of cortisone upon skin sensitivity to tuberculin was investigated in 14 patients with sarcoidosis. Ten patients who normally failed to react to at least 100 units of tuberculin intradermally received 100 mg. of cortisone daily by intramuscular injection. In 8 of these, skin sensitivity to tuberculin was detected at intervals ranging from six to seventeen days after the first injection of cortisone; in 6 the reaction produced by tuberculin during the period of sensitivity persisted, with induration, up to forty-eight hours; and in 2 it consisted of erythema, evident at twenty-four hours but fading at forty-eight hours. In those patients tested repeatedly, this sensitivity was transient, even though the cortisone was continued. In 9 patients who normally did not react to at least 100 units, intradermal injection of 1.25 mg. of cortisone with a dose of tuberculin equal to the greatest to which the patient had previously failed to respond produced a reaction with induration and erythema which persisted at least forty-eight hours in 7, with no response in 2. In 3 patients normally sensitive to 100 units or less, similar local administration of cortisone with tuberculin exhibited the reaction produced by tuberculin alone (Authors' summary).—*Effect of cortisone upon skin sensitivity to tuberculin in sarcoidosis, D. A. Pyke & J. G. Scadding, Brit. M. J., November, 22, 1952, No. 4794: 1126.—(E. A. Riley)*

Tuberculosis After Resection for Other Disease.—Two patients who had recently undergone lobectomy for bronchiectasis developed far advanced pulmonary tuberculosis. Most other cases of this sort have been reported in the statistics pertaining to resections for bronchiectasis. In three series, pulmonary tuberculosis developed in 3 to 13 per cent of the patients. In almost every reported case, review of the preoperative films showed no evidence of pulmonary tuberculosis. Apparently there were gross tuberculous changes in the unremoved lung tissue remaining after lobectomy. Distention of remaining pulmonary tissue, containing hidden unhealed tuberculous foci, is the most probable cause of the postoperative disease. All post-lobectomy patients should be followed with frequent chest roentgenograms to detect any development of pulmonary tuberculosis. When pulmonary tuberculosis is present in a patient who requires pulmonary resection for nontuberculous disease, surgery should be planned to avoid a situation conducive to overdistention. Thus, after lower lobe resection, phrenic paralysis, possibly associated with a period of pneumoperitoneum, would be desirable. When the disease processes are bilateral, prolonged sanatorium treatment of the tuberculous process with appropriate chemotherapy should be the method of choice.—*Pulmonary tuberculosis following resection for nontuberculous disease, E. Rothstein & C. E. Gerson, J. Thoracic Surg., June, 1952, 23: 575.—(R. E. MacQuigg)*

Lobectomy with Thoracoplasty.—Eleven male patients undergoing upper lobectomy in combination with a five-rib thoracoplasty had much less respiratory efficiency than that found immediately following each stage of thoracoplasty alone. The stress placed on the body by the combination of lobectomy and thoracoplasty was greater than that imposed by each stage of thoracoplasty alone. There was one postoperative death; the patient's most significant feature was the great increase in respiratory rate and in the dead-space ventilation and the decrease in tidal exchange. Autopsy revealed no cause of death but the physiologic alterations probably explain it. Only patients with good respiratory reserve should have a combination of thoracoplasty, which greatly cripples the efficiency of the respiratory mechanism, and pulmonary resection, which imposes a considerably greater load upon the body than does thoracoplasty alone. —*Physiologic studies following thoracic surgery: II. Immediate effects of upper lobectomy combined with a five-rib thoracoplasty, W. W.*

Stead, J. Thoracic Surg., February, 1953, 25: 194.—(R. E. MacQuigg)

Late Results of Thoracoplasty.—A series of 278 tuberculous patients was reviewed five to fifteen years after thoracoplasty. The series was typical with regard to sex and site of operation. No streptomycin or penicillin was used before or after operation. Of the entire series, 27 per cent of the patients have died. Of the 203 living patients, 85.7 per cent have "negative" sputum. When the 8 patients who died from nontuberculous causes with "negative" sputum are added, the figure is 89.6 per cent of the 211 patients. According to the 1944 National Tuberculosis Association classification, 140 of the 278 patients were apparently cured; 9 arrested; 36 apparently arrested; 2 were quiescent; 11 improved; 5 worse; and 75 dead. Haight, in discussing the paper, pointed out that these patients were operated upon, not because it was believed that they would obtain a good result, but in many instances because nothing else could be done.—*Results in 278 patients who had the modern type of thoracoplasty for tuberculosis, W. M. Lees, S. C. H. Yang, M. Papoulakos, J. Alexander, & A. Larralde, J. Thoracic Surg., October, 1951, 22: 329.—(R. E. MacQuigg)*

Late Results of Thoracoplasty.—Of 334 patients who received thoracoplasty for pulmonary tuberculosis from 1932 to July of 1948, 230 were living and 104 were dead from all causes by the end of 1949. Of the entire series, 58 per cent attained arrest or apparent cure of the disease. Among the 230 living patients and 12 others who died from causes unrelated to their arrested tuberculosis, 84.3 per cent had arrested or apparently cured tuberculosis. Late results in thoracoplasty justify its prominent place in the surgical treatment of pulmonary tuberculosis.—*Late results in the treatment of pulmonary tuberculosis by thoracoplasty, G. C. Adie, W. G. Childress, H. J. Brezing, & D. Taylor, J. Thoracic Surg., January, 1952, 23: 92.—(R. E. MacQuigg)*

Post-Thoracoplasty Painful Back.—Some patients with post-thoracoplasty back pain do not have impingement of the scapula upon the rib below but have overriding of the medial border of the scapula on the vertebral column because of atrophy of the trapezius and rhomboid muscles. In 5 of the latter sort, a flap of the trapezius and rhomboid muscles was raised from the opposite side of the chest, turned back on itself, and attached to the free edge of the muscle remaining on the medial border of the scapula on the side of the thoracoplasty. In 3 others a muscle flap was obtained from the under surface of the scapula and consisted of the subscapularis and serratus anterior muscles. This flap was attached to the free edge of the flap from the opposite side. All of the 8 patients were relieved of their symptoms, and there has been no atrophy of the muscle flaps or further interference with the range of motion. In one of the patients, resection of a portion of the scapula had been done to relieve pain and had failed completely, although the surgical procedure described gave him complete relief.—*Surgical treatment of post-thoracoplasty painful back, L. M. Shefts, J. Thoracic Surg., June, 1952, 23: 602.—(R. E. MacQuigg)*

CLINICAL STUDIES, NONTUBERCULOUS DISEASE

Purpura and Sarcoidosis.—A sixteen-year-old girl developed gingival bleeding, purpura, and thrombocytopenia. Her spleen was barely palpable, and a chest roentgenogram showed massive bilateral hilar adenopathy. Sternal bone marrow revealed normal erythropoiesis and myelopoiesis, megakaryoblasts in normal numbers, megakaryocytes increased, a large percentage showing basophilic cytoplasm, and no thrombocytes. Intramuscular corticotropin only slightly increased the circulating platelets, but bleeding ceased. Accordingly, on the sixth day of therapy a splenectomy was performed. Thereafter a slow rise in the platelets occurred. Follow-up examination six months later revealed a normal platelet count with nearly complete disappearance of the hilar lymph nodes. The appearance of the spleen was consistent with sarcoidosis. It is premature to assume a causal relation between sarcoidosis and thrombocytopenia, particularly since the

combination is not common despite the frequent involvement of the spleen in sarcoidosis. —*Thrombocytopenic purpura with sarcoidosis, cured after splenectomy, H. Schrijver & P. H. M. Schillings, Acta med. Scandinav., 1952, 144: 213.*—(*K. T. Bird*)

Nocardiosis.—A 37-year-old white man was explored for possible pulmonary neoplasm and found to have nocardiosis. He survived following drainage of an empyema cavity and intensive sulfonamide therapy; he was living and well a year later. The disease is caused by an aerobic gram-positive partially acid-fast *Actinomyces*. Of 43 cases in the literature including this one, only 8 can be considered to have recovered; all of these received sulfonamide therapy in one form or another. It is essential that these patients receive adequate dosage of sulfonamides and be maintained on therapy for four to six months.—*Nocardiosis, R. G. Connar, T. B. Ferguson, W. C. Sealy, & N. F. Conant, J. Thoracic Surg., October, 1951, 22: 424.*—(*R. E. MacQuigg*)

Emphysema.—Among 103 cases of emphysema in 76 men and 27 women, observed over a five-year period, only 3 gave any history of familial respiratory difficulties. Chronic bronchitis, sometimes with bronchospasm, was considered the cause in 50 patients (49 per cent), 41 of whom were men. Since cough and sputum preceded the dyspnea in most cases, chronic bronchitis seemed the primary disorder. In 19 cases asthma was the cause. The mean age of the bronchitic group was fifty-two years, that of the asthmatics was thirty-nine years. Pneumonia appeared to be the precipitating cause in 10 patients; 9 others were gassed in the first World War, and in one other man symptoms developed following exposure to a high concentration of sulfur dioxide fumes. Another 3 patients had silicosis, 7 had bronchiectasis, and emphysema developed in 4 without any apparent cause. Although the degree of emphysema could be determined more readily and accurately by clinical evaluation and spirometry, the most reliable radiologic signs were depression and flattening and diminished respiratory excursion of the diaphragms with hypertranslucency of the lung fields, especially at the bases and anterocardiac and retrocardiac windows. The heart shadow was characteristically small, the cardiothoracic ratio was reduced, and air was often visible between the diaphragm and the cardiac apex. These changes probably resulted from the low diaphragm and more vertical position of the heart. Two-thirds of the patients had an enlarged pulmonary conus, and one-fifth had right ventricular enlargement; but these changes were unrelated to the severity of the emphysema. Spirometrically, there were decreased vital capacity, increased residual air and its ratio to the total capacity, slowed expiration, and an increased mixing time in functional residual capacity determinations. The degree to which the maximum breathing capacity was reduced was a better guide to the severity of emphysema than were the changes in lung volume. Because of inadequate alveolar ventilation, the alveolar tension of oxygen was reduced and that of carbon dioxide raised. Although the exact pathogenesis of emphysema is obscure, one essential feature is atrophy of the lung's elastic tissue. The other important abnormality is rupture of alveolar septa with thinning and avascularity of the alveolar walls. The functional defects arise partly from the loss of elastic recoil and partly from the increase in alveolar space. Reduced diaphragmatic and lower costal activity with increased activity of the upper costal and accessory muscles of respiration are additional factors. Pulmonary hypertension secondary to the mechanical reduction of the vascular bed and also to the vasoconstriction following alveolar anoxia is an important factor in the development of secondary cardiac failure. The degree of emphysema was largely dependent upon the severity and duration of the primary causal disease. Although there is no curative treatment for the emphysema itself, symptomatic therapy was of marked value. Postural drainage and appropriate antibiotic therapy, either systemically or by inhalation, were valuable in controlling infection. Bronchodilators diminished dyspnea and increased exercise

tolerance when bronchospasm was present. Intermittent oxygen therapy relieved cyanosis and dyspnea but should not be employed continuously for fear of improving oxygen saturation, depressing ventilation, and causing further retention of carbon dioxide. Smoking was reduced as much as possible. If possible, patients were sent to a warm dry climate for the winter months. Breathing exercises were most beneficial in the younger age groups. The use of morphine is dangerous because of its powerful respiratory depressant action.—*Emphysema, A. G. W. Whitfield, Brit. M. J., December, 6, 1952, No. 4796: 1227.—(E. A. Riley)*

Mucoid Impaction of Bronchi.—Ten patients with asthma or chronic obstructive bronchitis had a distinct but undescribed clinical entity, mucoid impaction of the bronchi, which may closely simulate neoplasm, abscess, or tuberculosis. It results when inspissated mucus accumulates in the second-order branch bronchi distal to a bifurcation. As the laminated impaction grows, layer by layer, it dilates the bronchus to many times its normal diameter. When the neighboring branch bronchus is compressed, it also accumulates a mucoid mass. The dilated bronchial wall becomes infected and the cartilaginous elements are almost entirely destroyed. At operation the true diagnosis should be established before too much pulmonary tissue is resected. Hemotherapeutic agents, expectorant cough mixtures, and bronchodilators should be tried; bronchoscopy is usually not helpful. Persistent suppuration with destruction of pulmonary tissue, repeated hemoptyses from associated cystic bronchiectasis, and the necessity for excluding neoplasm may all require operation. The underlying asthma may lead to repeated episodes in other segments of the lung; resection should be limited to the actual lesion to preserve pulmonary function. At exploration it may be necessary to expose the bronchial wall to exclude neoplasm. With mucoid impaction the tumor is entirely within the bronchus; the bronchial wall is reddish-brown, edematous, and partially necrotic. The prognosis of this complication of asthma is the prognosis of the underlying disease. In 4 of the 10 patients, pneumonitis has recurred in other segments and undoubtedly was due to mucoid impaction.—*Mucoid impaction of the bronchi, R. R. Shaw, J. Thoracic Surg., August, 1951, 22: 149.—(R. E. MacQuigg)*

Pneumothorax in Purulent Pleurisy.—Two cases of purulent pleurisy became suddenly complicated by pneumothorax. They illustrate situations in which relatively simple surgical procedures are valuable. The first patient, a 3-year-old boy, was hospitalized for cough and a pain in the right side. His illness began with diarrhea about two weeks earlier, followed by symptoms of "grippe," in the course of which the child suddenly became worse. On the day he was admitted, a chest roentgenogram showed fluid and air in the right pleural space. Yellow pus containing *Staphylococcus aureus* was aspirated and a bronchopleural fistula was suspected. It was decided to do a costotomy with closed drainage under water. Twenty-four hours later, the fluid and air had completely disappeared and the patient left the hospital the next day, thirty-six hours after admission. He remained well, and six months later a roentgenogram showed only a faint pleural shadow. The second patient, a boy eighteen months old, was admitted because a pulmonary infection had suddenly become worse. A roentgenogram taken the next day showed beginning pneumothorax together with annular shadows suggesting cysts. Subsequent films showed an increasing pneumothorax. Pus was aspirated, presumably from one or more infected cysts. Radical surgical intervention was contraindicated by the age and the serious condition of the child. The air and pus were quickly drained through an ordinary intravenous needle inserted in the thoracic wall. The child recovered.—*Deux cas de pneumothorax a soupape, J-M. Lemieux & R. Thibaudeau, Laval med., March, 1953, 18: 302.—(A. T. Laird)*

Bronchogenic Carcinoma.—Among 1,357 men with lung cancer, only 7 (0.5 per cent)

had never consistently smoked as much as one cigarette a day for as long as a year. Among the same number of control patients with other diseases, 61 (4.5 per cent) were non-smokers. Of the group with carcinoma 25 per cent had smoked 25 or more cigarettes daily, or the equivalent in pipe tobacco, for at least ten years in comparison with 13 per cent of the controls. Among 108 women with lung cancer, 37 per cent were non-smokers compared with 55 per cent of the controls; 11 per cent had smoked 25 or more cigarettes a day compared with 1 per cent of the controls. No appreciable differences were found between patients with lung cancer and other patients in the use of lighters (43 and 41 per cent) or in the use of hand-rolled cigarettes (21 and 19 per cent). Five per cent of the patients with lung cancer regularly used a cigarette holder compared with 12 per cent of the controls, and only 3 of 504 smoked filter-tipped cigarettes compared with 15 of 467 controls. The lower proportion of pipe smokers (4 per cent) among men with lung cancer compared with a 7 per cent incidence of male controls who smoked suggests partial removal of some active agent before it reaches the respiratory tract. There was no statistical difference of patients who inhaled in the control and lung carcinoma groups. No correlation was noted with histologic types, although the total number of proved cases of adenocarcinoma, 43, was too small to conclude that no difference exists. No responsible agent in tobacco smoke has been detected.—*A study of the aetiology of carcinoma of the lung, R. Doll & A. B. Hill, Brit. M. J., December 13, 1952, No. 4797: 1271.*—(*E. A. Riley*)

Bronchial Fibrosarcoma.—Primary fibrosarcoma of the lung is rare, and such a lesion arising in a bronchus has been reported in only a few instances; diagnosis by bronchoscopic biopsy was accomplished in only 7 previously reported cases. Because these lesions are rare, no plan of therapy can be based on experience. The malignancy of the lesion and the impossibility of determining bronchoscopically the extent of its penetration into the lung suggest that pulmonary resection is the therapy of choice. The tendency of fibrosarcoma to be relatively radioresistant strengthens this opinion. Three cases of fibrosarcoma of the bronchus were diagnosed by bronchoscopic biopsy. One patient with a primary bronchial fibrosarcoma, seen relatively early and treated by resection limited to the right middle and lower lobes, was alive and apparently well thirteen months after operation. Fibrosarcoma of the bronchus, in common with lesions of this type encountered elsewhere in the body, is said to be a slowly growing tumor which spreads principally, if not exclusively, by local invasion; personal and reported experiences lend little support to that viewpoint. With exceptions, this entity has proved to be highly fatal, usually causing death two years or less after the time symptoms first appear.—*Fibrosarcoma of the bronchus, C. F. Storey, J. Thoracic Surg., July, 1952, 24: 16.*—(*R. E. MacQuigg*)

Tracheal Myxochondroma.—A patient with tracheal obstruction by a benign myxochondroma was treated by tracheotomy followed by resection of the involved tracheal wall. A full-thickness skin graft taken from the inner aspect of the right arm was reinforced with No. 28 gauge stainless steel wire, after the manner of Gebauer, and sutured into the defect so that airtight closure was accomplished. Re-examination one year later showed normal vocal cords and a widely patent trachea.—*Myxochondroma of the trachea, B. H. Evans, J. Thoracic Surg., December, 1951, 22: 585.*—(*R. E. MacQuigg*)

Reconstruction of Trachea and Bronchi.—The best reconstruction method for the trachea or bronchi is excision of the damaged or constricted area and anastomosis. This is facilitated if partial pulmonary resection is also necessary. Of the available autografts, wire-supported skin is best, and its clinical usefulness has been proved. Bronchial autografts are ideal, but their use is limited by their availability and unique clinical circumstances. Tubular prostheses provoke the formation of

a fibrous cylinder all around them, and their use may be only temporarily successful.—*Reconstructive surgery of the trachea and bronchi: Late results with dermal grafts, P. W. Gebauer, J. Thoracic Surg., December, 1951, 22: 568.*—(*R. E. MacQuigg*)

Mediastinal Liposarcoma.—Both a lipoma and a liposarcoma were completely removed from the superior mediastinum. Three months later the patient was asymptomatic and had essentially negative roentgenograms. This is the sixth case of solitary liposarcoma confined to the mediastinum. Thoracotomy usually is mandatory for accurate histologic diagnosis. All unilateral circumscribed mediastinal tumors should be explored since approximately 25 per cent of these tumors are malignant. Many originally benign lesions tend to become malignant and even benign tumors can cause serious illness or death by pressing on adjacent structures. Patients with liposarcoma have a doubtful prognosis as the tumor may recur locally, sometimes after a long interval.—*Liposarcoma of the mediastinum, C. F. Storey & K. P. Knutson, J. Thoracic Surg., September, 1951, 22: 300.*—(*R. E. MacQuigg*)

Unusual Mediastinal Cyst.—A benign mediastinal cyst resembled clinically a bronchiogenic carcinoma with mediastinal invasion. It decreased remarkably in size with radiotherapy and nitrogen mustard injections, especially the latter. This decrease in size could be attributed to the vomiting and dehydration caused by the nitrogen mustard or to the shrinkage by radiation therapy of the lymphatics which filled the cyst. As doubts of the diagnosis grew, the patient was readmitted for right exploratory thoracotomy, and a cystic cavernous lymphangioma, 10 cm. in the longest diameter, was found between the superior vena cava, the azygos vein, and the esophagus. Nearly a year later the patient was working full time with no symptoms referable to the chest. Exploratory thoracotomy seems indicated for any patient thought to have a bronchiogenic carcinoma with a paralyzed vocal cord unless definite evidence of tumor exists elsewhere.—*Case report: An unusual mediastinal cyst, E. F. Skinner, H. Isbell, & D. Carr, J. Thoracic Surg., May, 1952, 23: 502.*—(*R. E. MacQuigg*)

Congenital Esophagobronchial Fistula in Adults.—Congenital tracheo-esophageal or broncho-esophageal fistulas in adults have been proved in only 4 cases. Another occurred in a 40-year-old woman who had had a productive cough since early childhood. A broncho-esophageal fistula was demonstrated with barium. At operation a fistula was found between the esophagus and the basal bronchus of the left lower lobe, which was divided. Although lower lobectomy and lingulectomy had been planned, they could not be done because the patient's condition deteriorated. Eighteen days later the incision was reopened and a patent ductus was divided between clamps. Left pneumonectomy was performed; during it, an artery was found to arise from the aorta immediately above the site of the fistula and to communicate with the basal branch of the pulmonary artery. Complete recovery followed. Perhaps many so-called spontaneous fistulas in adults are really congenital, although symptoms may be delayed because a thin membrane persists for a time or because a valvelike fold prevents pulmonary aspiration.—*Congenital esophagobronchial fistula in an adult, J. K. Berman, P. S. Test, & B. A. McArt, J. Thoracic Surg., November, 1952, 24: 493.*—(*R. E. MacQuigg*)

Esophageal Achalasia.—In 1927, Rake showed that the cells of Auerbach's plexus in the lower esophagus are absent or degenerate in patients with achalasia. The scarcity of ganglion cells correlated with the degree of achalasia. The terminal esophagus in some patients with achalasia dilates like a sphincter under the influence of amyl nitrite and ergotamine; hypertrophied circular muscle fibers in the distal 2 or 3 cm. of the esophagus act like a sphincter. The scarcity or absence of ganglia cells in the terminal esophagus may be congenital or developmental. Psychogenic causes have been suggested for the achalasia but the

emotional problems of these patients soon disappear when normal eating is restored and are likely due to their esophageal dysfunction. Most of the patients do tend to be apprehensive and introspective. Only those therapeutic measures which enlarge the inferior esophageal segment and cardia have succeeded. Most surgeons who employ the Finney type of cardioplasty have stated that there is more symptomatic than objective evidence of relief. The Heineke-Mikulicz procedure gives both symptomatic relief and objective evidence that the dilated esophagus becomes smaller. Although the patient is able to swallow, regurgitation esophagitis and gastritis with secondary bleeding and anemia are common. After Heineke-Mikulicz or Finney cardioplasties, 9 of 32 patients had esophageal bleeding and anemia. In 4 of the patients, bleeding was severe. All of the 9 patients had to follow a careful ulcer regimen even though there was no hyperchlorhydria. Since better long-range results might follow resection of the lower esophagus, the cardia, and fundus of the stomach, 3 patients were so treated. For two years all were able to eat satisfactorily, but one followed an ulcer regimen because of regurgitation esophagitis and hemorrhage one month postoperatively. The results were no better than after a modified Heller operation, which is considerably less extensive. During the past year 6 patients were operated upon by a modified Heller technique. There was no evidence of postoperative esophagitis and bleeding. Wangensteen, in discussing the paper, made a plea for earlier operative myotomy if one or two attempts at dilatation failed. He excised the distal segment, together with the entire acid-secreting area of the stomach, only for a tortuous, baglike esophagus. A coincidental Ramstedt procedure was done on the pyloris to ensure satisfactory gastric emptying after excision of the vagi which necessarily attends the procedure. Mega-esophagus, congenital hypertrophic pyloric stenosis, and mega-colon may be related entities.—*Observations as to the etiology and treatment of achalasia of the esophagus, E. B. Kay, J. Thoracic Surg., September, 1951, 22: 254.—(R. E. MacQuigg)*

Dilatation for Cardiospasm.—Cardiospasm (achalasia, mega-esophagus, or preventriculosis) is a nonorganic stenosis of the lower esophagus and is seldom more than 4 to 6 cm. long. Diffuse spasm is a separate entity which occasionally coexists with cardiospasm but does not respond to treatment in the same manner. The two entities may readily be differentiated by passing an olive-tipped bougie over a previously swallowed thread. Plummer's hydrostatic dilator also is passed over a thread. Nearly all patients get a thread in place if they persist. When the thread is firmly anchored, a 41 French olive-tipped metal bougie attached to a flexible whalebone staff is passed into the stomach. A 50 or 60 F. Plummer sound is then guided over the thread by a flexible wire spiral. Following the passage of these sounds, the patient rests for forty-eight to seventy-two hours. The Plummer dilator is passed again just after a 41 olive which defines the exact level of the obstruction. Once in place the bag is carefully inflated to 22 to 24 feet of water pressure and the pressure maintained for several seconds. The beneficial effects are immediate. If complete relief from dysphagia is not obtained, a second hydrostatic dilatation is performed forty-eight or seventy-two hours after the first. Two or three hydrostatic dilatations usually suffice. Of 601 cases of cardiospasm so treated, 205 had had some previous dilatation. An esophagoscope had to be used to aid the dilatation of 39 patients; 46 were treated by sounds without hydrostatic dilatations. Of the 555 new patients treated with the hydrostatic dilator, 452 could be followed. A satisfactory result with dysphagia or only transient difficulty for at least four years was obtained in 313 cases (69.2 per cent). Patients who did not respond satisfactorily to the initial course were less likely to obtain good results following a second course (38.3 per cent) or from additional courses (19.1 per cent). Patients with pulsion diverticula of the esophagus were more difficult to treat and had poor results, as did

those with hiatal hernia. Hiatal hernias of the short esophagus type were particularly troublesome and dilatation of the cardiospasm in such cases was hazardous, 10 cases of esophageal rupture occurring near the cardia. Of these patients, 2 died of mediastinitis and a third had empyema requiring surgical drainage. In another case, the lower part of the esophagus and the upper part of the stomach were resected and the patient did well. The remaining 6 patients recovered completely with conservative therapy. Operative treatment should be considered whenever hydrostatic dilatation or more conservative measures do not give satisfactory results, which is true for approximately 20 per cent of the patients. Most of the procedures permit regurgitation of gastric secretions causing inflammatory changes in the lower esophagus. No definite conclusions can be drawn from the 23 cases treated surgically because eight different operative procedures were used. The Heller operation appears to be best; it retains some of the cardia's sphincteric function. After an operation which destroys sphincteric action, the gastric acidity should be reduced.—*The treatment of cardiospasm: Analysis of a twelve-year experience, A. M. Olsen, S. W. Harrington, H. J. Moersch, & H. A. Andersen, J. Thoracic Surg., August, 1951, 22: 164.*—(*R. E. MacQuigg*)

Mercury-Bag for Passing Esophageal String.—Graduated dilators guided through the esophagus by passage over a slightly tensed, previously swallowed string were introduced by Mixter in 1909 and are now in common use for a variety of esophageal strictures. A rubber finger cot containing 2 cc. of mercury was used to anchor the string on twelve occasions. The method is fast, safe, and reliable as a means of carrying the string down the gastrointestinal tract, and not to serve as a bougie. The use of a swallowed string for esophageal dilatation is contraindicated in the presence of an anatomic loop, such as a gastroenterostomy or enteroenterostomy, at any level in the alimentary tract, for the string may circle the loop and ulcerate the mucosa as reported by Hubbard and Leven.—*Mercury-bag bolus to expedite passage of esophageal dilator guide string, F. H. Aves, G. Desforges, & J. W. Strieder, J. Thoracic Surg., July, 1952, 24: 34.*—(*R. E. MacQuigg*)

Acute Cardiac Arrest.—In acute cardiac arrest, the quickest approach should be used for manual compression of the heart. Usually this will be by a direct transthoracic approach. Artificial circulation and efficient artificial ventilation allow time for additional therapy. This consists of intravenous or intra-arterial transfusions if the blood volume has been depleted. A patient who has been hypoxic for a long period may suffer irreparable damage to the brain in much less time than the arbitrary three and one-half minutes. Harken, in discussing the paper, cited work of Claff and Dickson which indicated that the best defibrillating equipment must be capable of giving one or two brief shocks of 140 volts at 3 amperes. Bailey stressed that success in cardiac resuscitation depends upon the training of the anesthetist to recognize the condition and upon prompt thoracic intervention. He used epinephrine, 0.5 cc. initially, to restore myocardial tonus, if there was ventricular fibrillation which did not respond to electric shock, and to improve the heartbeat after overcoming the standstill. The minimum current that will defibrillate an adult ventricle is 110 volts at 5 to 10 amperes. Potts stressed the prevention of these accidents by proper anesthetic techniques because inadequate exchange of gases and excessive anesthetic agents are the cause of idiopathic cardiac arrest.—*Analysis of twenty-seven cases of acute cardiac arrest, J. L. Ehrenhaft, D. W. Eastwood, & L. E. Morris, J. Thoracic Surg., December, 1951, 22: 592.*—(*R. E. MacQuigg*)

Removal of Cardiac Lipoma.—A primary lipoma was removed from the heart, apparently for the first time. It is the second instance of the successful removal of any cardiac tumor. The tumor presented is the largest primary one, malignant or benign, described in the literature. Only 2 proved cases of pri-

mary lipoma of the heart have previously been reported.—*Successful removal of tumor of the heart, E. R. Maurer, J. Thoracic Surg., May, 1952, 23: 479.—(R. E. MacQuigg)*

Surgery for Pulmonary Stenosis.—In 30 consecutive cases of the tetralogy of Fallot, direct intracardiac relief of pulmonary stenosis was attempted. The stenosis was thought to be valvular in 15, infundibular in 12, and localized to the pulmonary artery in 3. The currently accepted procedures for relieving pulmonary stenosis in the tetralogy are dangerous because an additional vascular defect is produced. The direct approach would appear to offer a more satisfactory long-term solution. Some cases will require a shunt, but these should be selected only after thorough intrapericardial assessment of the anatomic situation.—*The direct intracardiac relief of pulmonary stenosis in the tetralogy of Fallot, R. B. Glover, C. P. Bailey, T. J. E. O'Neill, D. F. Downing, & C. R. E. Wells, J. Thoracic Surg., January, 1952, 23: 14.—(R. E. MacQuigg)*

Pulmonary Stenosis.—Since the operative procedures for correcting infundibular stenosis and pulmonary stenosis are different, increasing importance has been attached to the preoperative differentiation of these conditions. During cardiac catheterization, fluoroscopic observation of the catheter tip and continuous pressure determinations will allow anatomical localization of the site at which the change from high right ventricular pressure to low pulmonary arterial pressure occurs. Under ideal conditions it can be determined whether this change occurs abruptly in the region of the pulmonary valve. In infundibular stenosis it is frequently possible to detect an intermediate zone of pressure in the outflow tract of the right ventricle which is characteristic of infundibular stenosis. The absence of this finding does not necessarily mean, however, that infundibular stenosis is not present. The magnitude of pressure differences between the infundibular region and right ventricle proper depends upon the degree of infundibular stenosis relative to that present at the valve. If the stenosis is mild or localized close to the valve, the intermediate high pressure zone may not be apparent.—*The problem of isolated valvular versus infundibular pulmonic stenosis with particular reference to cardiac catheterization data and records obtained at the time of operation, D. C. Connolly, R. Lev, J. W. Kirklin, & E. H. Wood, Proc. Staff Meet., Mayo Clin., February, 11, 1953, 28: 65.—(E. A. Riley)*

Pulmonary Valvulotomy.—The pulmonary valvulotomy described by Brock was modified by using the midline sternal-splitting approach instead of the left anterior intercostal approach requiring manual displacement of the heart into the left thorax. The procedure was performed in 8 patients. Good functional results were obtained even when the right ventricular pressure was not elevated to extreme levels and when mild pulmonary hypertension was present. No deaths occurred.—*Pulmonary valvulotomy, H. B. Shumacker, Jr. & P. R. Lurie, J. Thoracic Surg., February, 1953, 25: 173.—(R. E. MacQuigg)*

Results of Blalock Shunts.—Among 200 patients with congenital heart disease treated by Blalock-Taussig anastomotic operations, more than 80 per cent had tetralogy of Fallot and gave the best results. In the series, 75 per cent of the patients were between the ages of five and fourteen years. The diagnosis was usually based upon clinical examination. Angiocardiography helped in diagnosing doubtful cases and in deciding upon operation. Cardiac catheterization showed evidence of a right to left shunt by pressure determinations and oxygen studies. The usual operative procedure was an end-to-end anastomosis of the left subclavian artery to the left pulmonary artery. The follow-up period varied from one to five years. Good results were obtained in 68 per cent; 17 patients were improved, 17 showed no change, and there were 24 (12 per cent) operative deaths. Among this group, 13 patients had some lesion other than an uncomplicated tetralogy and 5 were over the

age of nineteen. All except one died within seventy-two hours of operation. Postoperatively a murmur could be heard resembling that of a patent ductus arteriosus; a thrill was unusual. Clubbing regressed but seldom disappeared. Cyanosis improved, and the hemoglobin concentration tended to fall toward normal. Cardiac enlargment occurred fairly rapidly after operation. Patients with large hearts preoperatively did not do so well as those with hearts of normal size. Too large an anastomosis was suspected in patients whose hearts continued to enlarge after the initial increase. Congestive failure was then a serious possibility. Symptoms improved immediately after operation and subsequent deterioration or evidence that the shunt was decreasing in size was rare. The radial pulse disappeared on the operative side. Horner's syndrome and minor cerebral thrombosis, both uncommon, have been the only postoperative complications.—*Results of the Blalock-Taussig operation in 200 cases of morbus caeruleus, M. Campbell & D. Deuchar, Brit. M. J., February 14, 1953, No. 4806: 349.—(E. A. Riley)*

Mitral Stenosis.—Surgical treatment of mitral stenosis was carried out in 13 patients. Most of them had marked invalidism not relieved by adequate medical treatment. The results were good to excellent in 10 patients, 2 were failures, and there was one death. Apical systolic murmurs were unchanged postoperatively in 5 patients, more marked in 2, and less marked in 2, while the intensity of the diastolic murmur decreased in 8 patients. When successful, the operation resulted in decreased pressures in the pulmonary circulation, increased cardiac output, reduction of the heart size, and a remarkable improvement in functional capacity. The chief indication for operative treatment is predominant mitral stenosis with little or no mitral insufficiency, aortic valvular disease, or severe myocardial disease, as determined by clinical examination, roentgenograms, and cardiac catheterization. Operation has not been performed in patients with minor symptoms or in the absence of an increase in the pressure in the pulmonary circuit. Mitral valvulotomy is contraindicated in clinically active rheumatic carditis or subacute bacterial endocarditis and in marked cardiac insufficiency, right heart failure, marked mitral insufficiency, or aortic valvular disease. Neither auricular fibrillation nor considerable cardiac enlargment is a serious contraindication. Digital rupture of the stenosed valves is generally easy to perform at the commissures, the site of preference. The mitral orifice should preferably be dilated to permit the passage of one and one-half to two fingers. If an adequate opening is not secured, there will be less benefit from the operation and a greater risk of postoperative thrombosis in the left auricle. A successful slit in the stenosed valves is generally followed by a reduction in pressure and size of a tense left auricle, while, at the same time, the small left ventricle expands, demonstrating powerful, sweeping contractions instead of the previous small and rapid beats. The picture may even give the impression of imminent cardiac dilatation but, provided routine preoperative digitalization has been complete, further observation will disprove this impression.—*Studies in mitral stenosis: I. Results of one year's series of surgically treated cases, H. B. Wulff, G. Biörck, N. P. Bergh, H. Krook, O. Axén, & O. Lundskog, Acta med. Scandinav., 1952, 144: 275.—(K. T. Bird)*

Commissurotomy for Mitral Stenosis.—The first 256 consecutive commissurotomies for mitral stenosis were reviewed. The operative mortality for the 199 cases in 1951 and 1952 was 4 per cent. The youngest patient was four years of age and the oldest, fifty-nine, with an average age of thirty-five years. Of the series, 164 patients have been followed for six months to three and one-half years. At least six months must elapse before the patient can appreciate the subjective benefits, and a longer period is often required for objective changes to stabilize. In 21 patients, cerebral, visceral, or peripheral emboli had occurred before operation. Of these, 14 had auricular fibrillation but only 9 had gross thrombi in the left auricular appendage at operation. No

embolic episodes have occurred postoperatively in any of these 21 patients. The relief of left atrial stasis by opening the valve is at least as important as the ligation of the base of the left auricular appendage in achieving this result. Auricular fibrillation is not a contraindication to surgery unless the ventricular rate is entirely uncontrollable. In 69 patients with auricular fibrillation, the eventual results compared favorably with those in patients with sinus rhythm. Cardiac catheterization of 14 patients five to twenty-three months after surgery showed that the pressure changes in the first postoperative month do not necessarily reflect the ultimate pressure. Despite the pathologic nature of the valve, satisfactory separation of the valve leaflets was accomplished in 157 cases. Valve function could not be restored in approximately 25 cases of fibrotic scarring and calcification which destroyed the pliability of the cusps. Use of the guillotine commissurotomy knife was necessary in approximately 30 per cent of the cases. In an additional 20 to 40 per cent, a more thorough commissurotomy could be performed. Two patients awoke from anesthesia with hemiplegia. A third, who had had many emboli before operation and whose appendage contained a clot, developed pain in the popliteal area shortly after surgery. Eventually a mid-thigh amputation was necessary and the patient recovered. No fresh emboli could be found in the amputated limb, the vessels of which were thickened and contracted as in thromboangiitis obliterans. No exacerbations of acute rheumatic fever occurred but pleuropericarditis, which may have been rheumatic, appeared transiently during the first six postoperative months in 63 patients. Although patients referred for surgery late in the course of their disease have been restored to a high level of efficiency and enjoy a more normal life, they must always remain under a physician's care. The selection of patients for operation at an early age is a responsibility of both physician and surgeon, who can no longer avoid or postpone it. In discussing this paper, Black agreed with Bailey that the *bête noir* of mitral surgery is the production of emboli, either of blood clots or calcium particles from the valve; temporarily obstructing the arteries in the neck rather than in the chest may have reduced the hazard of embolism.—*The indications for and the results of commissurotomy for mitral stenosis, R. P. Glover, T. J. E. O'Neill, J. S. C. Harris, & O. H. Janton, J. Thoracic Surg., January, 1953, 25: 55.*—*(R. E. MacQuigg)*

Constrictive Pericarditis.—A patient with combined constrictive pericarditis and constrictive pleuritis was successfully treated by pericardiectomy and bilateral pulmonary decortication. The constrictions were apparently the result of a crushing injury to the chest with uninfected bilateral hemothorax and hemopericardium. Discussion of this paper revealed divergent opinions in regard to the importance of releasing various areas of the heart. E. Holman stated that he had produced all of the symptoms of cardiac compression by constricting the right heart alone; constricting the left heart alone gave no symptoms of the usual type. Overholt recalled that Schmieden in 1926 pointed out that the left ventricle must be freed completely. There has since been disagreement as to whether the other portions of the heart should be liberated. Two years earlier, most of the discussants had favored extending decortication to include the vena cava but Dexter, by cardiac catheterization, demonstrated patients in whom the difficulty was entirely due to left ventricular constriction. Sweet never saw evidence of obstruction on the right border of the heart. Woodbury catheterized 8 patients and never found that the pressure gradient increased between peripheral vein and right atrium; thus vena caval constriction did not cause the elevated peripheral venous pressure. Hansen of Copenhagen catheterized 10 patients with constrictive pericarditis and never found pressure gradients in the great vessel indicating localized constriction at any point. Thus the elevated venous pressures must be due to impaired ventricular function. This interference is not confined to the right ventricle alone since pulmonary capillary and pulmonary

arterial pressures were elevated in each of the 18 cases. Woodbury was convinced by this evidence that the left ventricle should be completely liberated whenever pericardiectomy is performed for constrictive pericarditis.—*Constrictive pericarditis and constrictive pleuritis treated by pericardiectomy and pulmonary decortication, C. S. Burwell, J. W. Woodbury, & J. H. Walker, J. Thoracic Surg., January, 1952, 23: 1.*—(*R. E. MacQuigg*)

Restoration of the Coronary Circulation.—In coronary arteriosclerosis the lumina of the large and medium-sized coronary arteries are progressively occluded by subintimal thickening. The smaller branches without muscular coats are spared. Hence, an adequate collateral blood supply might be developed through these smaller branches. Only a small percentage of the normal coronary flow can be replaced from extracardiac sources by suturing adjacent vascular tissues to the heart or by producing vascularized adhesions between pericardium and epicardium with talc or asbestos. Arterialization of the coronary sinus to reverse the flow of blood through it was suggested by Roberts and was carried out in animals and in patients by Beck and his coworkers. Communications between the superficial cardiac and deep myocardial veins normally drain blood through small openings into certain cardiac chambers. These communications permit arterial blood entering the coronary sinus to escape by way of the myocardial veins. This blood is lost from the capillary bed and does not nourish the cardiac muscle but the communications afford a valuable safety mechanism regulating the retrograde flow without defeating the purpose of the arterialization. Injection studies of the hearts of 3 dogs following the Beck procedure demonstrate that the branches of the coronary sinus establish large epicardial anastomoses with the small cardiac and anterior cardiac veins of the right ventricle. The left side of the heart and septum possesses a much richer arterial and venous system than does the right side. The coronary sinus is continuous with capillaries of the left ventricle and septum; it also has several collateral channels leading to the lumen of the right atrium. The myocardium is better nourished after the Beck procedure because: (*1*) arterial blood flows retrograde through the extensive venous tributaries of the coronary sinus, providing new avenues to the capillary bed; and (*2*) branches of the coronary vessels anastomose with such extracardiac arteries at the base of the heart as those of the pericardium, vasa vasorum of the aorta and pulmonary vessels, and new arterioles growing into the wall of the vein graft. A recent myocardial infarction contraindicates surgery for at least six months; the operation must be deferred until the patient's electrocardiograms have been invariable for four to six months. Two myocardial infarctions within one year or multiple infarctions are contraindications. Congestive heart failure or gallop rhythm is an absolute contraindication. The myocardial damage usually is too extensive to be aided by operative interference when patients have suffered congestive failure on the basis of coronary artery disease. For technical reasons, a markedly calcified aorta is a contraindication. Digitalization prior to surgery is not helpful and may initiate troublesome arrhythmias or conduction defects. In the original Beck technique, heavy plastic ligatures passed around the coronary sinus approximately one centimeter from its terminus are left loose about the sinus. One end of a segment of a large superficial vein, previously removed for grafting, is then sutured into the side of the coronary sinus proximal to the loose ligatures. A special toothed clamp is used to isolate a portion of wall without interrupting the aortic blood flow. The vein graft is then anastomosed to this isolated area. Three weeks later, the former incision is opened and the plastic ligatures are tightened over a 3 mm. probe laid against the coronary sinus. When the sound is withdrawn, some of the sinus blood can escape into the right atrium; most of the blood flows from the aorta through the vein graft into the sinus and its tributaries. The Beck procedure was modified by excising a button of aortic wall rather than making a longitudinal incision; this prevents or delays

thrombosis of the vein graft. Of the 18 patients operated upon, 2 died: one from a sudden hemorrhage at the side of the aortic anastomosis on the seventh day; the other a few hours after surgery, apparently from circulatory depression. Thrombosis of the anastomosis was found in 7 patients at the time of the second operation. The other 7 patients have improved, their status ranging from "clinical cure" to moderate amelioration of symptoms. Objective evaluation of progress in cases of coronary disease is always difficult. Reversing the blood flow through the coronary sinus so changes the electrocardiogram that it is valueless as a measure of improvement in these cases. The theoretical advantages and the subjective improvement of these patients are great enough to warrant further use of the operation.—*The anatomic (histologic) basis and efficient clinical surgical technique for the restoration of the coronary circulation, C. P. Bailey, R. C. Truex, A. S. Angulo, G. D. Geckeler, W. Likoff, N. Antonius, & W. B. Neptune, J. Thoracic Surg., February, 1953, 25: 143.*—(*R. E. MacQuigg*)

Wedge Pressures.—In patients studied by direct recordings of pressure at operation, tracings of pressure made when the catheter tip was wedged in a small pulmonary artery have been found to correlate closely both in pressure and in contour with recordings of left atrial pressures made simultaneously. The oxygen saturation of blood withdrawn from the catheter with the tip in the pulmonary-artery wedge position usually exceeds the saturation of systemic arterial blood; this difference is exaggerated when low mixtures of oxygen are breathed. On occasion, venous blood can be obtained when the tip of the catheter is in the wedge position. The recording of pressures with the catheter tip in the pulmonary-artery wedge position has been free from complications in all of the 181 patients from whom such tracings have been obtained. Satisfactory mitral commissurotomy usually is followed by an immediate decrease in left atrial and pulmonary-artery pressure, of approximately the same magnitude. The contours of pulmonary-artery wedge pressure cannot be considered an infallible index as to the presence or absence of mitral insufficiency (Author's summary).—*Pulmonary-artery wedge pressures in mitral valve disease: Relationship to left atrial pressures, D. C. Connolly, R. G. Tompkins, R. Lev, J. W. Kirklin, & E. W. Wood, Proc. Staff Meet., Mayo Clin., February 11, 1953, 28: 72.*—(*E. A. Riley*)

Oximeter Test of Pulmonary Function.—The earpiece oximeter and a basal metabolism machine may be used to evaluate the efficiency of pulmonary gas exchange. After obtaining the basal saturation of the patient while he breathes room air, the blood's oxygen saturation is lowered to 80 per cent by having him re-breathe into the air-filled spirometer. The patient is then quickly switched to room-air breathing and the time for the saturation to rise from 80 to 90 per cent is measured. Measurements on 55 healthy adults indicated a normal re-saturation time in this test of 13.4 ± 3.4 seconds. Tests on 300 patients with pulmonary tuberculosis but without clinical cyanosis demonstrated deficiencies in 53 per cent. In 38 per cent of the patients exhibiting deficiencies, it was possible to estimate whether the deficiency was predominantly due to reduced ventilation or to impaired gas diffusion. Re-testing of the patients reproduced the results well enough to allow the test to be used routinely for evaluating patients. In several cases, the oximeter has established the presence or absence of a pulmonary basis for a complaint of dyspnea. Unlike ventilatory tests, these oximeter readings cannot be distorted by lack of effort, willful or otherwise, on the part of the patient.—*A simple test of pulmonary function employing the oximeter, R. S. Alexander & M. M. Reydman, J. Thoracic Surg., January, 1953, 25: 95.*—(*R. E. MacQuigg*)

Body Fluids and Electrolytes During Thoracic Surgery.—To determine changes in the body fluids and mineral metabolites of patients undergoing thoracic surgical procedures, 20 unselected patients were studied pre- and

postoperatively. They received no parenteral therapy other than blood replacement during the operation and maintained adequate urinary outputs postoperatively, despite low oral fluid intake. Sodium retention was as marked following thoracic procedures as after other surgical procedures; the 24-hour sodium excretion decreased from an average of 195.5 mEq per day preoperatively to an average of 69.7 mEq per day up to the fourth postoperative day. Serum chloride and sodium concentrations as well as carbon dioxide combining power remained within normal limits. Serum potassium and urinary potassium excretion were relatively unaffected by uncomplicated thoracic surgical procedures. Underestimation of the blood replacement required at operation largely explained the low plasma volume and total circulating protein in the early postoperative period. In this study, blood loss was estimated with T-1824 blue dye and by the Wangensteen "dry weighted sponge" method. The extracellular fluid compartment markedly expanded postoperatively in 17 of the 20 patients. In the presence of normal serum sodium levels, this increase in extracellular fluid regularly paralleled salt retention, which explains the extracellular fluid increase. Increased extracellular fluid postoperatively is dangerous for thoracic surgical patients with poor pulmonary reserve, pulmonary hypertension, and anoxia. Infusions, particularly of saline solutions, may produce detectable edema. Saline solutions should not be administered to a thoracic surgical patient unless hyponatremia or water intoxication exists.—*Body fluid shifts, sodium and potassium metabolism in patients undergoing thoracic surgical procedures, E. M. Aronstam, C. H. Schmidt, & E. Jenkins, Ann. Surg., March, 1953, 137: 316.*—(*M. J. Small*)

Severe Irradiation Damage of Lung.—In each of 2 patients with typical radiation damage of one lung, disabling cough and dyspnea were markedly relieved by pneumonectomy. In the specimens, the hyaline membranes were absent. It is believed that they are an artifact. Adams, in discussing the paper, commented that some roentgenologists believed that associated infection was an important contributing factor and gave antimicrobial treatment during radiotherapy and the posttherapy period of maximum reaction. One patient, considered inoperable because of low pulmonary function, improved greatly on prolonged antimicrobial therapy. In cases of carcinoma of the breast, esophagus, or other adjacent malignancy where the lung may be incidentally irradiated, high voltage radiation may reduce lung damage. Brantigan and Macht have used Dicumarol as anticoagulant therapy during radiation therapy. None of the patients so treated showed radiation fibrosis of the lung on autopsy, even though the primary carcinoma receded or almost completely disappeared.—*Pneumonectomy for severe irradiation damage of the lung, M. Bergmann & E. A. Graham, J. Thoracic Surg., December, 1951, 22: 549.*—(*R. E. MacQuigg*)

Delayed Roentgenogram in Bronchography.—In mapping the extent of surgical resection for a patient with bronchiectasis, a film taken some minutes after bronchography may be valuable. Filling an additional segment may reveal definite bronchiectasis or may avoid needless sacrifice of tissue by eliminating uncertainty. When iodized oils are used, excess medium in the proximal bronchus shows that enough contrast substance is present for complete visualization. Retained secretions, material trapped by a stricture, the kinking of a bronchus, or air trapped locally as the contrast substance flows in may each delay the passage of iodized oil. A delayed roentgenogram is indicated when the wet film taken immediately following bronchography discloses adequate contrast substance in a proximal bronchus but none in its peripheral branches. Before the additional film is taken, the patient should remain about five minutes in a position which favors the medium's flowing into the unfilled segments; in this position he should breathe slowly and deeply to encourage maximum dilation and elongation of the bronchi as a further aid to entrance of the oil. Such delayed films have avoided the need for later instillations of

oil and have eliminated transient hydrostatic bronchial dilations which might be mistaken for minimal bronchiectasis. The delayed roentgenogram will not obviate the need for expectorants, antispasmodics, postural drainage, or bronchoscopy when these appear indicated as preliminaries to bronchography.—*Indications for a delayed roentgenogram in bronchography, I. J. Kane & S. Heiser, J. Thoracic Surg., July, 1952, 24: 70.—(R. E. MacQuigg)*

ANTIMOCROBIAL THERAPY

Isoniazid in Pulmonary Tuberculosis.—Twenty patients with pulmonary tuberculosis were observed for a ten-week period while being treated with a daily oral divided dose of 250 mg. of isonicotinic acid hydrazide. All patients had received prior courses of combined streptomycin and para-aminosalicylic acid (PAS); 13 had previously undergone major thoracic surgery. At the end of four weeks of therapy, the number of organisms per specimen was significantly reduced among 16 patients with sputum consistently positive for tubercle bacilli on smear and on culture prior to treatment. At the end of ten weeks, 12 of the 16 "positive" cases had become "negative" on direct smear. Cultures with one exception remained positive for tubercle bacilli. Cough and volume of sputum were reduced. Cavitation was present in 11 patients. One patient had cavity closure in eight weeks; cavities decreased in size in 3 patients, increased in size in one, and showed no change in 7 patients. Improvement of other potentially reversible lesions was noted in 7 patients. A spread occurred in one, and there was no significant roentgenographic change in the remaining patients. Contrary to experience elsewhere, hemoglobin levels definitely tended to increase. Most patients noted improved appetite and sense of well-being; fever and toxic symptoms usually cleared within a few days. Side effects of the drug were unimportant. One patient developed a moderate hemoptysis. Clotting time was prolonged and plasma prothrombin activity was reduced but returned to normal within two weeks after isoniazid was discontinued. In 2 patients with a prior history of hemoptysis but not included in the series, hemorrhage occurred while isoniazid was being given, but hematologic studies showed no abnormality. Because of the possibility of adverse reactions such as hemoptysis, this drug, particularly if higher doses are employed, should be given only in a hospital.—*Isonicotinic acid hydrazide in pulmonary tuberculosis, A. D. Lapp, G. A. Black, & J. N. O'Rafferty, Canad. M.A.J., December, 1952, 67: 619.—(E. A. Riley)*

Isoniazid.—Isoniazid was given by mouth to 196 patients with pulmonary tuberculosis for 120 days in doses of 150 or 300 mg. daily, alone or combined with 2 gm. of streptomycin given intramuscularly every third day. A preliminary evaluation of the 104 patients who completed 120 days of treatment on these regimens indicates that isoniazid is an effective agent for the treatment of pulmonary tuberculosis. It did not cause serious toxicity in this dosage during the first four months of therapy. The effectiveness of the drug was slight in this dosage when employed alone, and drug-resistant organisms emerged rapidly in a high percentage of patients. Limited data suggested that the combination of isoniazid and streptomycin enhanced the therapeutic effectiveness and delayed the emergence of tubercle bacilli resistant to both agents. Final evaluation of isoniazid will depend on a more complete analysis based on a large series of patients treated for eight to eighteen months with continuous combined drug therapy (Authors' summary).—*Isoniazid therapy in pulmonary tuberculosis: A preliminary report, F. W. Pitts, F. L. Miller, W. E. Dye, C. W. Tempel, & M. J. Fitzpatrick, U. S. Armed Forces M. J., January, 1953, 4: 1.—(E. A. Riley)*

Isoniazid and Carbohydrate Metabolism.—When reducing substances were found in the urine of 5 of 24 tuberculous patients receiving isoniazid, oral glucose tolerance tests were used to determine whether isoniazid produced any disturbance in the carbohydrate metabolism in 6 diabetic tuberculous patients

and 6 nondiabetic tuberculous patients. The results indicate that the administration of isoniazid in therapeutic doses produces a temporary elevation of the blood sugar and makes both the rise and fall of an oral glucose tolerance test slower in both diabetic and nondiabetic patients. In the nondiabetic control patients, a mean rise of blood sugar of approximately 20 mg. per 100 ml. over three hours was observed; the rise in diabetic patients was spectacular over a short period in some cases. The insulin requirements of diabetic patients who receive isoniazid thus may be increased.—*Effect of isoniazid on carbohydrate metabolism in controls and diabetics, G. R. W. N. Luntz & S. G. Smith, Brit. M. J., February 7, 1953, No. 4805: 296.*—(*E. A. Riley*)

Isoniazid in Guinea Pigs.—Isoniazid was given to guinea pigs infected with a streptomycin-sensitive strain of *M. tuberculosis* H37Rv three weeks previously and, hence, with well-established experimental tuberculosis. The dose of isoniazid was 4 to 6 mg. per kg. daily for ninety-four days. All isoniazid-treated animals survived until the end of the experiment and, in 50 per cent of them, it was impossible to diagnose tuberculosis macroscopically. The remaining animals showed only minimal lesions. Evaluation of the degree of gross tuberculosis, considering 100 to represent maximal involvement, gave 70 for the untreated control group, 23 for the streptomycin-treated group, and 8 for the isoniazid-treated group. Another series of animals was infected with a bacillary strain resistant to streptomycin and sensitive to *p*-aminosalicylic acid (PAS). The degree of tuberculous involvement was 79 in the controls, 66 in the streptomycin group, 70 in the sodium-PAS group, and 13 in the isoniazid group.—*Régression des lésions anatomiques de la tuberculose experimentale du cobaye traitée par l'isoniazide, J. Baudot, Delande, Moreau, & Serena, Presse med., February, 1953, 61: 288.*—(*V. Leites*)

Tuberculous Meningitis.—Thirty-three patients with tuberculous meningitis have been treated with streptomycin and the survivors have been re-examined from four to five and a half years after the institution of treatment. Nine (27 per cent) are alive and well and leading normal lives, 3 are gravely disabled, and 21 have died after periods ranging from two days to sixty months. Spinal block occurred in 9 of the patients; 7 are dead, and a partial block persists in one of the 2 survivors. Intracranial calcification as seen in skiagrams has occurred in 10 of the 13 patients who survived long enough for it to develop. Six of these are well, 2 are disabled, and 2 have since died. Relapse of meningitis has occurred in 9 patients. Only one of these children is alive and well, 2 others are disabled, and 6 have died of the relapse (Authors' summary).—*Tuberculous meningitis treated with streptomycin: A four-year follow-up, S. J. M. Russell & P. MacArthur, Brit. M. J., January 24, 1953, No. 4803: 192.*—(*E. A. Riley*)

PAS and Thyroid Enlargement.—A case of thyroid gland enlargement with exophthalmos followed treatment by *p*-aminosalicylic acid (PAS). It is suggested that PAS produces its effect by binding iodine and so preventing the iodization of tyrosine. It may thus have a goitrogenic effect similar to that of thiouracil (Author's summary).—*Effect of PAS on the thyroid gland, R. R. Hamilton, Brit. M. J., January 3, 1953, No. 4800: 29.*—(*E. A. Riley*)

Pyrazinamide.—Pyrazinamide (Aldinamide) has been used in 19 cases of tuberculous lymphadenopathy with draining sinuses. Patients ranged in age from seventeen months to sixty-seven years. Twelve patients were treated without operative surgery other than repeated aspirations of fluctuant masses in three instances. Seven patients were submitted to excisional surgery for the removal of diseased tuberculous nodes and sinuses. Treatment extended for periods of eleven to ninety days, averaging approximately seventy days in the unoperated group and twenty-two days in the operated group. The dosage schedule was 1 gm. thrice daily for all except the smaller children and one elderly patient to

whom reduced doses were given. Follow-up periods after stopping pyrazinamide in the nonoperated group are short, the maximum being approximately three months and the average only slightly over eight weeks. In only one instance has there been any evidence of recurrent activity. Two of the operated group developed signs of reactivation, one at the operative site and one adjacent to the operative site following trauma. This may indicate the advisability of continuing pyrazinamide for longer periods after surgery. One patient in the group noted gastrointestinal upset with some dizziness.—*Use of pyrazinamide (Aldinamide) in the treatment of tuberculous lymphadenopathy and draining sinuses, J. W. V. Cordice, L. M. Hill, & L. T. Wright, J. Nat. M. A., March, 1953, 45: 87.*—(*L. Hyde*)

Plasma Levels of Viomycin.—Twenty patients with pulmonary tuberculosis received single intramuscular injections of viomycin sulfate, and the resulting plasma levels were determined by an agar diffusion assay technique. Ten of the patients received 1 gm. and ten 0.5 gm. of the drug. Blood was drawn before and at intervals of one, two, four, and eight hours thereafter. With both dosages the peak plasma levels were obtained one or two hours after the drug had been administered. All 10 patients receiving the 1.0 gm. dose had maximum plasma levels ranging from 61 to 118 γ per ml. Nine of the 10 on the 0.5 gm. dose had peak plasma concentrations of 22 to 43 γ per ml.; the levels in the tenth patient remained below these values.—*Plasma levels of viomycin in man after single intramuscular injection, W. E. Dye & W. F. Bull, Am. J. Clin. Path., January, 1953, 23: 56.*-(*J. S. Woolley*)

TB 3.—A short clinical trial of para-ethylsulfonylbenzaldehyde thiosemicarbazone (TB 3) was made in 8 patients who suffered from tuberculosis of the spine or sacroiliac joint. The first 4 patients were started on 50 mg. daily and gradually increased to 200 mg. daily in divided doses. The later cases were started on 100 mg. daily and raised more rapidly to 200 mg. daily. No attempt was made to assess therapeutic effects since serious toxic reaction forced abandonment of the clinical trial in its early stages. Among these side effects were transient albuminuria without evidence of permanent renal damage, the appearance of reducing substances in the urine, anorexia, nausea, vomiting, and anemia. —*Clinical trial of TB 3 in surgical tuberculosis, J. H. Mayer & P. G. Laing, Brit. M. J., January 24, 1953, No. 4803: 189.*—(*E. A. Riley*)

Antituberculosis Activity of Quinolines.—Nineteen quinoline derivatives, including eleven 3-amino quinoline derivatives, were evaluated for tuberculostatic action upon *M. tuberculosis* H37Rv. *In vitro*, the most active derivatives of 3-amino quinoline and beta-naphthylamine were those most easily hydrolyzed. Hydrazine derivatives were usually less active. *In vivo*, the sulfonamide derivative showed some therapeutic effect for the tuberculous guinea pig but was found to be toxic. The therapeutic results compared unfavorably with those of streptomycin and *p*-aminosalicylic acid.—*Chemotherapy of experimental tuberculosis with quinoline derivatives, B. L. Freedlander, A. Furst, & D. Balcom, Proc. Soc. Exper. Biol. & Med., October, 1952, 81: 66.*—(*E. Soto-Figueroa*)

LABORATORY STUDIES

Rapid Demonstration of M. tuberculosis in Egg-Yolk Sac.—Acid-fast strains of *M. tuberculosis* were readily demonstrated by smear and by subsequent guinea pig inoculation of the yolk sac contents of five- to eight-day embryonated eggs which had been inoculated only four to six days previously with suspected specimens of spinal fluid, pericardial or pleural exudates, gastric washings, sputum tissues, and exudates. Of 39 specimens derived from patients with various types of tuberculous infection and tested in this way, 39 were proved by guinea pig inoculation to have *M. tuberculosis* and of 11 known negative controls none were "positive." The method provides a rapid diagnostic method.—*Isolation of M. tuberculosis by in-*

oculation of the yolk sac of embryonated eggs, J. W. Brueck & G. J. Buddingh, Proc. Soc. Exper. Biol. & Med., August–September, 1952, 80: 589.—(F. B. Seibert)

Laryngeal and Pharyngeal Swabs.—Of 100 patients with pulmonary tuberculosis in Montevideo, 46 failed to yield *M. tuberculosis* by any method of specimen collection. Conventional sputum and gastric cultures yielded positive results in 44 per cent, whereas combined pharyngeal and laryngeal swab techniques increased the percentage to 52. The laryngeal swab method proved more sensitive than the pharyngeal swab but 9 cases were "positive" with the pharyngeal swab alone, and the highest percentage of positive results was obtained using both methods. The swab technique gave negative results in only 2 cases which were "positive" with the routine methods. Cultures obtained with laryngeal and pharyngeal swabs showed a very low incidence of contamination (1.63 per cent). The method is simple, rapid, can be performed at any time of the day, and repeated if necessary. It gives much less discomfort to the patient than gastric lavage.—*Recherches sur la culture du bacille de Koch dans les mucosités laryngo-pharyngéennes prélevées par la methode du frottis, A. Saenz, Presse med., March, 1953, 61: 381.—(V. Leites)*

Immunization with Living Tubercle Bacilli.—There are no simple, dependable techniques for assessing the protective effects of vaccination against tuberculosis. Development of tuberculin allergy may indicate that the vaccine has "taken," but allergy is no index of the amount of immunity present. A high level of allergy can exist without immunity to infection and vice versa. There is no present technique for the passive transfer of antituberculosis immunity or for its study *in vitro*. Even with favorable circumstances, the immunity produced by injecting attenuated bacilli is relatively weak, and many animals must be tested for dependable results. In the present experiment, mice were vaccinated intravenously with small doses of attenuated BCG-T or BCG-P or of avirulent H37Ra tubercle bacilli. They were challenged with the highly virulent MV human strain two or more weeks later. The vaccinated mice rapidly developed partial resistance against infection with virulent bacilli, as indicated by (*1*) prolonged survival of the vaccinated animals after a dose of virulent bacilli which killed all of the unvaccinated controls in ten to twenty days, and (*2*) fewer bacilli cultured from the spleen and lungs of vaccinated animals after a small dose of virulent bacilli. The level of immunity depended upon the extent to which the bacilli of the vaccine multiplied *in vivo*. This in turn was dependent upon the degree of attenuation of the bacterial strain used for the vaccine, the amount of vaccine injected, the route of vaccination, and the time interval between vaccination and the challenge infection. Isoniazid prevented or retarded the development of immunity when the mice were treated during immunization. Although immunity regularly developed and lasted for many weeks when the proper conditions of vaccination were used, the immune response never protected the animals against death from infection with virulent tubercle bacilli. The lives of the vaccinated mice were not prolonged by a direct bactericidal effect but rather by retarded or interrupted multiplication of the virulent bacilli *in vivo*.—*Antituberculous immunity induced in mice by vaccination with living culture of attentuated tubercle bacilli, R. J. Dubos, C. H. Pierce, & W. B. Schaefer, J. Exper. Med., February 1, 1953, 97: 207.—(J. S. Woolley)*

Immunization with Killed Tubercle Bacilli.—Definite antituberculous immunity can be induced in animals by vaccinating them with bacilli killed by heat, formaldehyde, ultraviolet radiation, et cetera. To define some of the factors which control the development of resistance in mice vaccinated with dead bacilli, vaccines prepared from strains of various degrees of virulence were compared and the influence of heat or chemical agents on the immunizing antigens was studied. Partial resistance to subsequent infection developed in

white mice following vaccination with small numbers of tubercle bacilli killed by contact with 2 per cent phenol. Vaccine prepared from avirulent H37Ra tubercle bacilli proved as active as vaccines prepared from virulent or attenuated bacilli. The immunity induced by phenol-killed bacilli persisted at least seven weeks but the virulent bacilli of the challenge infection were never killed, even when the challenge inoculum was small. As judged by prolonged survival of challenged mice, the protective effect of the vaccine could be detected even when the infecting dose was large. Fewer bacilli survived in the organs of vaccinated mice after a small challenge infection. The immunity produced in mice by phenol-killed bacilli was of the same order as that produced by live BCG. The protective antigen of the vaccine was destroyed by boiling, particularly in acid. It retained its activity when the bacilli were disintegrated and rendered nonacid-fast by grinding with concentrated phenol. Although present in the insoluble cellular debris, it was absent from the soluble components of the cells. Since some immunity can be established by injecting small amounts of dead bacillary substance, BCG vaccination may exert its protective effect, not because of some effect of the growing of the bacilli in the host, but merely by producing adequate amounts of bacillary substance during multiplication *in vivo*. Since the antigenic substance is present in avirulent as well as virulent killed bacilli, it is unrelated to any bacillary component responsible for virulence.—*Antituberculous immunity in mice vaccinated with killed tubercle bacilli, R. J. Dubos, W. B. Schaefer, & C. H. Pierce, J. Exper. Med., February 1, 1953, 97: 221.—(J. S. Woolley)*

Multiplication of Tubercle Bacilli in Mice.—The virulence of tubercle bacilli can be measured in terms of the severity and duration of the lesions produced in animals injected with known doses of the bacilli. It is still unknown whether bacterial variants differ in virulence because of their ability to multiply, or survive, or to exert toxic effects *in vivo*. The present experiments compared the rate and extent of multiplication *in vivo* of strains of tubercle bacilli which differ in their pathogenicity for experimental animals. The different strains were injected in various doses into mice, for the most part intravenously. By means of agar plate counts, the numbers of living bacilli in the lungs and spleen were determined at intervals after infection. Strains of the variant H37Ra did not multiply *in vivo*, although they survived for many weeks in the tissues of the mouse. These organisms are truly *avirulent*. Two BCG bovine strains and the R1Rv human strain multiplied *in vivo*, even when extremely small infective doses were used, but they did not give rise to progressive disease. Such strains which produce only abortive infections are *attenuated*. Different levels of attenuation were detected. The maximum numbers of living bacilli recovered from the tissues corresponded directly to the severity and duration of the abortive lesions and were characteristic for each strain. The two BCG strains differed markedly in their level of attenuation. Two human types (H37Rv and Amerzanga) and two bovine strains (Ravenel and MV) established progressive infection in mice even when small infective doses were used and are *virulent*. Attenuated and virulent strains at first were much fewer in the lungs than in the spleen but increased in the former organs throughout the period of observation. This was notably true in the case of the virulent cultures. The number of living bacilli in the spleen rapidly reached a maximum with all strains and then decreased progressively. For a given infective dose and a given interval after inoculation, the maximum numbers of living bacteria in the spleen and in the lungs proved to be a direct expression of the virulence of the strain.—*Multiplication and survival of tubercle bacilli in the organs of mice, C. H. Pierce, R. J. Dubos, & W. B. Schaefer, J. Exper. Med., February 1, 1953, 97: 189.—(J. S. Woolley)*

Effect of Vaccination on Intracellular Tubercle Bacilli.—Monocytes from peritoneal exudate can be infected with tubercle bacilli, either virulent or attenuated. The bacilli

multiply intracellularly when extracellular proliferation is prevented by small amounts of streptomycin in the liquid culture medium. With this technique the multiplication of tubercle bacilli was studied within monocytes derived from normal guinea pigs or rabbits and from animals previously vaccinated with BCG. The bacillary strains used for infecting the monocyte cultures were virulent H37Rv and Vallée and avirulent R1Rv and 2 strains of BCG. Tubercle bacilli multiplied abundantly within the cytoplasm of monocytes from normal guinea pigs or rabbits. By contrast, intracellular multiplication of the bacilli was retarded or completely inhibited within the monocytes of animals vaccinated with BCG. This inhibition of growth was observed with both virulent or attenuated strains of tubercle bacilli. The ability of monocytes to inhibit bacillary proliferation was the same whether serum from a normal animal or from vaccinated animals was used in the tissue culture medium. Moreover, the serum of vaccinated animals did not inhibit multiplication of tubercle bacilli within monocytes derived from a normal animal. The ability of guinea pig monocytes to interfere with intracellular bacillary proliferation was first perceptible eight days after vaccination and was complete at fifteen days. The multiplication of virulent and attenuated bacilli within the monocytes from the control animals at different periods was always uniform. These experiments suggest an active role for the monocytes in reinfection tuberculosis.—*Multiplication of tubercle bacilli within mononuclear phagocytes in tissue cultures derived from normal animals and animals vaccinated with BCG, E. Suter, J. Exper. Med., February 1, 1953, 97: 235.*—*(J. S. Woolley)*

Human Leukocytes and Tuberculin.—The effect of tuberculin and staphylococcal toxin on human leukocytes from tuberculous and nontuberculous subjects was studied. The method used to demonstrate cell injury was based on the fact that certain cellular contents are released into the surrounding medium when cells are damaged. The content selected for measurement is an enzyme with the properties of lysozyme. No significant difference could be detected in the degree of cell injury caused by exposing leukocytes from tuberculous or nontuberculous subjects to tuberculin for two hours *in vitro*. The same observation was made using staphylococcal toxin. A higher titer of lysozyme-like activity was found in the plasma obtained from tuberculous than from nontuberculous subjects. The factors governing the level of the lysozyme-like enzyme reported here are too little understood to afford an explanation of the observations.—*Plasma levels and the release of a lysozyme-like enzyme from tuberculin-exposed leukocytes of tuberculous and nontuberculous human beings, G. P. Kerby & S. N. Chaudhuri, J. Lab. & Clin. Med., April, 1953, 41: 632.*—*(F. G. Petrik)*

Serum Complement in Human Tuberculosis.—Fifty per cent units of complement determined on groups of normal individuals, tuberculous patients, and tuberculous patients receiving corticotropin (ACTH) showed highest activity in the tuberculous group and least in the normal group. Differences between the complement activity of normal and tuberculous individuals were significant. An increased complement activity (decreased 50 per cent unit) after long continued ACTH administration was observed. The association of an increased complement activity and a decreased gamma-globulin concentration was highly significant. No correlation was observed between complement activity and other electrophoretic components, namely, albumin, alpha-1 and alpha-2 globulins, beta globulin, and fibrinogen.—*Relation between serum complement and plasma electrophoretic fraction levels in human tuberculosis, G. Pitner & L. C. Smith, Proc. Soc. Exper. Biol. & Med., October, 1952, 81: 69.*—*(E. Soto-Figueroa)*

Hemagglutinins Produced by Stefanski's Bacilli.—The hemagglutination reaction performed with a carbohydrate antigen (*"antigène poliosidique"*) extracted from *M. tuberculosis* revealed the presence of antibodies in 15 of 18

rats eight to twenty-six weeks after subcutaneous infection with Stefanski's bacilli. The reaction was negative in 10 noninfected rats and in 3 infected rats treated with isoniazid. The fact that rats infected with Stefanski's bacilli form antibodies reacting with an antigen extracted from *M. tuberculosis* is a new argument for the kinship of these 2 microorganisms. The maximal titer of 1:16 is low, however, in comparison with titers obtained with serum from patients with tuberculosis and leprosy. It cannot be stated at present whether the low titer is caused by a great difference in the antigenic properties of Stefanski's bacilli and Koch's bacilli or by the inability of the rat to produce a high titer of antibodies (Author's summary).—*Réaction de Middlebrook-Dubos chéz le rat infecté par le bacille de Stefanski avec un antigène extrait du bacille de Koch, M. Viette, Ann. Inst. Pasteur, February, 1953, 84: 434.*—(*V. Leites*)

Continuous Versus Phasic Lung Inflation.—Mechanically imposed ventilation is often applied during thoracic surgery. Manual compression and release of the breathing bag may prevent respiratory acidosis. Controlled respiration, whether accomplished manually or automatically, in the absence of continuous and precise monitoring of arterial blood carbon dioxide pressure, pH, and oxygen tension is unlikely to be as responsive to the patient's needs as spontaneous respiration. An apparatus under the control of the subject's respiratory center has been devised. In dogs at least, the performance of the respiratory center remains satisfactory under light ether anesthesia. Continuous inflation of the lungs of dogs with their chests open for two hours or less is usually accompanied by substantial decline in cardiac output and the development of severe respiratory acidosis. These may be minimized or avoided by interrupting the positive respiratory pressure to permit passive exhalation against atmospheric pressure. The device described applies positive respiratory pressure during inspiration only and is controlled by the animal's own respiratory rhythm.—*Circulatory and respiratory effects of continuous versus phasic lung inflation in open-chest dogs, H. A. Frank, A. M. Cahan, & H. Banks, J. Thoracic Surg., May, 1952, 23: 465.*—(*R. E. MacQuigg*)

Bronchial Pulmonary Communications.—When the pulmonary artery and vein of a lobe in the dog's lung are both occluded, the vascular pressures within the lobe rise to abnormally high levels. This *in vivo* evidence supports the existence of functional communication between the bronchial and pulmonary arteries within the normal lung. The functional significance of the communications is speculative at present. Of greatest interest is the role they may play in the fate of a pulmonary segment or lobe deprived of its direct pulmonary arterial supply by embolus or other occlusive pathology. If such a segment had also a high degree of functional venous obstruction, as from chronic passive congestion or mitral stenosis, it is not improbable that bronchial collateral flow at systemic pressures would produce a destructive imbalance with capillary engorgement, edema of the alveoli and interstitial tissues, and frank hemorrhage. This may be the mechanism of hemorrhagic infarction.—*Observations on the hemodynamics of bronchial-pulmonary vascular communications, D. P. Shedd, R. D. Alley, & G. F. Lindskog, J. Thoracic Surg., November, 1951, 22: 537.*—(*R. E. MacQuigg*)

PUBLIC HEALTH AND EPIDEMIOLOGY

BCG Testing.—An intracutaneous injection of BCG vaccine may deviate from the recommended technique in 2 principal ways: more or less than 0.1 ml. may be given or the vaccine may not be injected superficially into the skin. In several studies, altogether comprising about 1,400 schoolchildren, the volume and depth of the BCG vaccination were deliberately varied within the limits likely to occur during ordinary mass vaccinations; the effect of such variations on post-vaccination tuberculin allergy and on local reactions at the site of vaccination was determined. Because most syringes leak, the volume of vaccine injected is commonly gauged by the size of the skin

wheal rather than by reading the graduated markings on the syringe. In approximately 950 children, different volumes of BCG vaccine were injected very superficially into the skin; 0.1 ml. of vaccine produced wheals averaging 8 mm. in diameter. Doubling or halving the volume caused a difference averaging 1.5 mm.; a sixfold increase (from 0.05 to 0.3 ml.) caused a 4 mm. increase in the diameter. In a second study of more than 400 children, the depth of injection was varied but the volume was kept constant. Deep intracutaneous injection of 0.1 ml. gave wheals averaging 3.1 mm. smaller than the same volume injected superficially, a decrease as great as that from a fourfold reduction in volume. Tuberculin allergy in the children was practically unaffected by variations in technique likely to occur in the field. Volumes of 0.05 to 0.3 ml. of vaccine, injected at depths ranging from very superficial in the skin to subcutaneous, all gave approximately the same mean level of post-vaccination allergy. A given volume of vaccine injected into the subcutaneous tissue just beneath the skin produced local lesions twice as large as those from the same volume injected superficially. The frequency of local subcutaneous abscesses increased with increasing depth of injection. These studies show that the size of the reaction at the site of vaccination is influenced by the depth of injection of vaccine, but the level of BCG-induced tuberculin allergy is not. If post-vaccination allergy is an index of immunity, the local vaccination reaction does not necessarily measure the same thing (Authors' summary).—*Variation in technique of intracutaneous BCG vaccination, C. E. Palmer & P. Q. Edwards, Brit. M. J., February 14, 1953, No. 4806: 363.—(E. A. Riley)*

Side Effects of BCG.—During a mass vaccination campaign conducted by the French Public Health Service in North Africa, few general reactions followed BCG administration. Vaccination during the pre-allergic phase or in the presence of positive tuberculin allergy produced an accelerated reaction at the site of injection. The site of a previously negative tuberculin test often flared into a spontaneous positive reaction. The rare complications in this group consisted of fever, malaise, and asthenia of several days' duration in the second week after vaccination. Some persons also developed around the vaccination area a maculopapular eruption which occasionally became generalized. The course of these complications was always benign and only about 10 such episodes were observed in approximately 10 million vaccinations. Other abnormal local reactions were: (*1*) an excessively large lesion, (*2*) abscess formation, and (*3*) abnormally slow healing. The eventual outcome was always benign in these cases. Painless enlargement of the lymph node draining the site of injection was found in 6 to 12 per cent of all vaccinated persons and subsided within two to four weeks. The adenopathy became purulent in 8.6 persons per thousand. Purulent adenopathy was directly proportional to the concentration of the vaccine and was always associated with a high degree of tuberculin allergy. It was more frequent during the hot season; more than half the cases occurred in children under two years of age. Population groups with poor hygiene had a particularly high incidence of this complication.—*Incidents et accidents observés après la vaccination, R. Mande, J. Gand, G. Houel, P. Babeau, & M. Gauthier, Semaine d. hôp. Paris, February, 1953: 648.—(V. Leites)*

Leaking Tuberculin Syringes.—An apparatus was devised to measure leakage between the plunger and barrel of tuberculin syringes. Even with the most satisfactory brand of syringe, approximately one of every 4 new syringes leaks to a greater extent than is compatible with accurate work. After being used for 1,000 to 1,500 intradermal injections, many syringes begin to show obvious signs of leakage, and, when retested, fail to meet the criteria for acceptability. Unless new syringes are tested before use and a plan for periodic retesting is followed, it is unreasonable to expect field workers to be able to measure accurately the amount of fluid given in intra-

dermal injections or to avoid wasting rather large amounts of tuberculin and vaccine (Authors' summary).—*Measurement of leakage of tuberculin syringes, J. Guld & C. Rud, Brit. M. J., February 14, 1953, No. 4806: 368.*—(*E. A. Riley*)

Tuberculin Tests in Schoolchildren.—During the period from February, 1947, to May, 1952, 7,415 tuberculin skin tests were given to 3,522 children in the schools of a semi-rural district near Brussels. Children aged three to six years showed 6.9 per cent positive reactions; children from seven to nine showed 16.4 per cent; children ten to twelve, 30.7 per cent; thirteen and fourteen, 40.7 per cent; fifteen and sixteen, 54.6 per cent. Of 100 children converting from negative to positive, 73 had no clinical manifestations; 27 had clinical incidents, less than half of which merited any notice. In 34 children, one or more incidents accompanied the skin test. There were 4 serofibrinous pleurisies, 3 parenchymatous consolidations, 2 cases of phlyctenular keratitis, 2 cases of erythema nodosum, one pneumonia, one hemoptysis, one case of meningitis (no tubercle bacilli were found in the spinal fluid), and 23 cases of swollen glands in the right or left hilar regions accompanied by increased parenchymous shadows in the area. In some of these cases the child had measles, whooping cough, or a severe cold at the time. Attempts to correlate the severity of the skin reactions with the exposure to tuberculosis in the home, with residence in a populous district, residence in the country, or with the number of children in the family were unsuccessful. Such studies may aid in the early detection of sources of tuberculosis and, if nationwide, might uncover most infectious or potentially infectious persons in less than five years.—*Le virage de la cuti-réaction à la tuberculine chez les enfants en âge scolaire, A. Jeurissen & Mme. Jeurissen-Lauwers, Acta tuberc. belg., December, 1952, 43: 485.*—(*A. T. Laird*)

Mass Roentgenograms.—Facilities for unlimited chest roentgenograms were offered to general practitioners in southwest London. Of the patients referred for examination because of minor complaints which did not justify referral to a chest physician, 2 per cent had active tuberculosis. A total of 3 per cent had radiologically significant lesions requiring further investigation; 48 per cent of the patients had minimal disease.—*Detection of tuberculosis in general practice by photofluorography, F. A. Nash, B. Benjamin, & T. J. Lee, Brit. M. J., February 7, 1953, No. 4805: 304.*—(*E. A. Riley*)

Tuberculosis Case Finding.—Two tuberculin surveys of infant schoolchildren were conducted to discover adult tuberculosis among the household contacts of tuberculin-positive children. In the first survey, 1,865 children were tuberculin tested by a chest physician and the contacts of positive reactors were examined by fluoroscopy. In the second survey, 1,933 children were tuberculin tested by school nurses and the contacts of positive reactors were examined by mass radiography. In both surveys, one new case of adult tuberculosis was found among the contacts for every 250 tuberculin tests applied in the schools. The cooperation of the mothers in persuading contacts to attend is an important factor in the success of the plan.—*Tuberculosis case-finding by the tuberculin-testing of infant-school children, I. A. MacDougall, J. R. Mikhail, & W. H. Tattersall, Brit. M. J., January 10, 1953, No. 4801: 64.*—(*E. A. Riley*)

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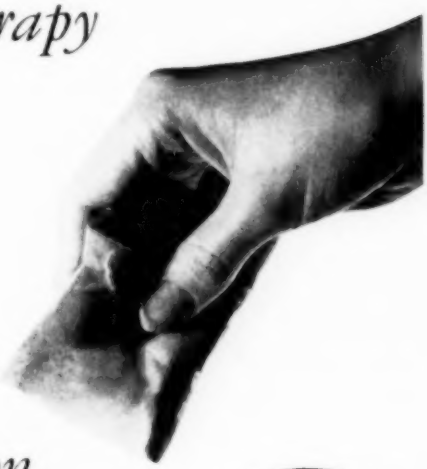

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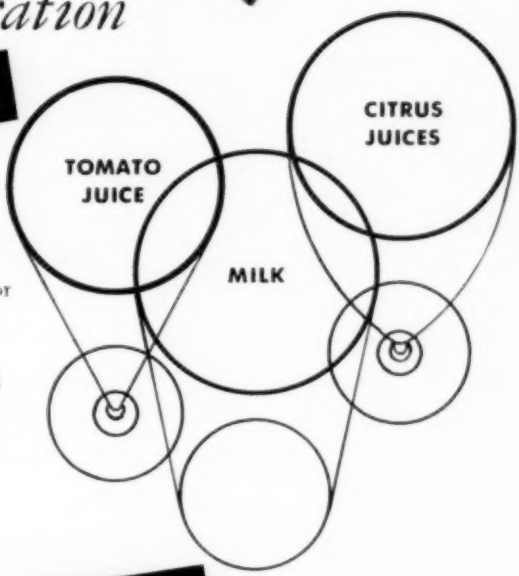

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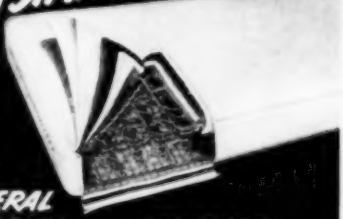

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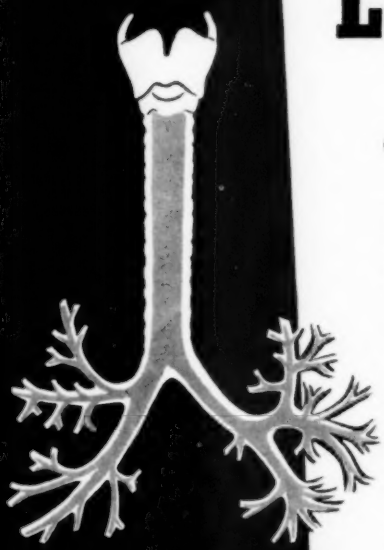

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1. Meakins, J. C.: The Practice of Medicine, ed. 5, St. Louis, The C. V. Mosby Company, 1950, p. 154.
2. *ibid.*, p. 268.
3. Bishop, P. A., and Lindskog, G. E.: Lung Abscess, in Pillmore, G. U.: Clinical Radiology, Philadelphia, F. A. Davis Company, 1950, vol. 1, p. 339.

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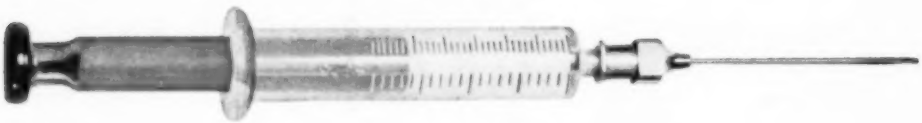

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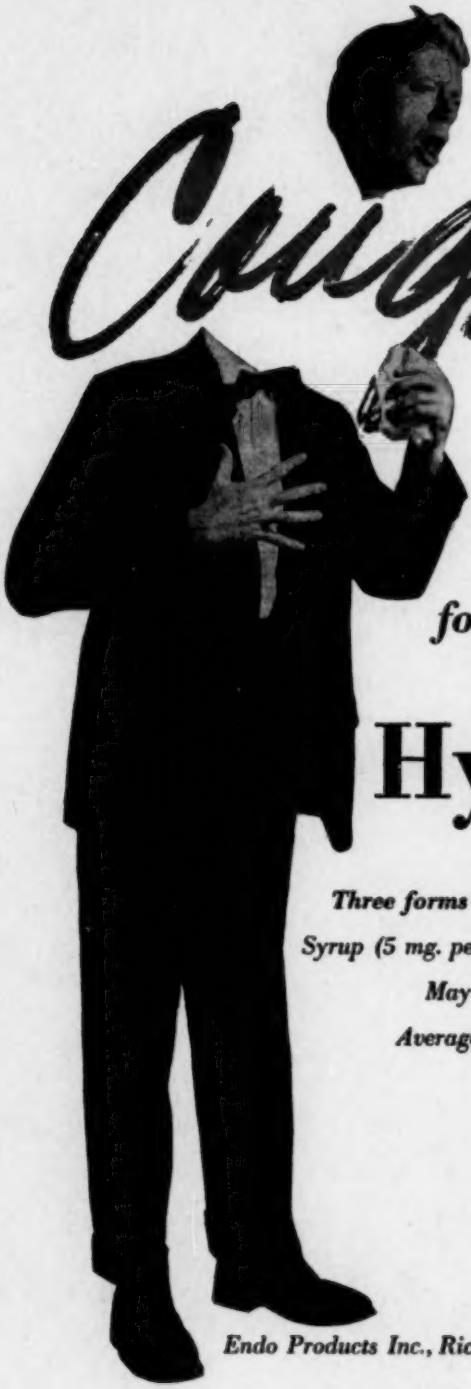

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